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(54) Title: OLIGONUCLEOTIDE THERAPEUTICS FOR TREATING HEPATITIS C VIRUS INFECTIONS

(57) Abstract: Short double stranded RNA (dsRNA) oligonucleotides homologous to regions of hepatitis C virus target RNA polynucleotide sequences are provided. Also provided are methods of attenuating the expression of hepatitis C virus genes, attenuating the function of hepatitis C virus target RNA polynucleotide sequences required for virus infection, replication, or pathogenesis, and otherwise inhibiting hepatitis C virus infection, replication, and/or pathogenesis by administering one or more of these short dsRNAs to prevent or treat hepatitis C virus infections in humans.

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OLIGONUCLEOTIDE THERAPEUTICS FOR TREATING HEPATITIS C VIRUS INFECTIONS

This application claims the benefit of priority of United States Provisional Patent Applications Serial Nos. 60/313,076, filed August 17, 2001; 60/344,116, filed December 20, 2001; and 60/353,750, filed February 1, 2002, the contents of which are each incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to compositions and methods for preventing or treating Hepatitis C Virus (HCV) infections in humans. These compositions comprise short double stranded RNA (dsRNA) oligonucleotides which, when introduced into cells, tissues, or organs where HCV is present, result in the inhibition of HCV infection, replication, and/or pathogenesis-associated phenomena. These dsRNAs presumably act by inhibiting the expression of HCV genes by degrading HCV genomic RNA, or other RNAs required for these processes, by the phenomenon variously known as RNA interference (RNAi) or RNA silencing.

Description of Related Art

Gene Silencing by Double Stranded RNA

"Gene silencing" or "RNA interference" (RNAi) is a mechanism of inhibiting gene expression that acts through a double stranded (dsRNA) intermediate, and results in sequence-specific targeting and degradation of the homologous messenger RNA (mRNA) (reviewed in Zamore, *Nature Structural Biol.* 8:746-750 (2001)). The proposed catalytic mechanism states that dsRNA, introduced as either a viral replicative intermediate or an artificial construct, is cleaved into fragments 25 base pairs long that target mRNA of homologous sequence for specific degradation. This phenomenon has been demonstrated in a number of organisms, including *Caenorhabditis elegans* (Fire et al., *Nature*

391:806-811 (1998); Montgomery et al., *Proc. Natl. Acad. Sci. USA* 95:15502-15507 (1998); PCT International Publication W099/32619); *Drosophila melanogaster* (Kennerdell et al., *Cell* 95:1017-1026 (1998)); *Trypanosoma brucei* (Ngo et al., *Proc. Natl. Acad. Sci. USA* 95:14687-14692 (1998)); planaria (Sanchez Alvarado et al., *Proc. Natl. Acad. Sci. USA* 96:5049-5054 (1999)); hydra (Lohmann et al., *Dev. Biol.* 214:211-214 (1999)); zebrafish (Wargelius et al., *Biochem. Biophys. Res. Commun.* 263:156-161 (1999); and embryonic mice (Wianny et al., *Nat. Cell Biol.* 2:70-75 (2000)). This phenomenon appears to be related to gene silencing phenomena ("cosuppression" or "post-transcriptional gene silencing (PTGS)) in plants (Vaucheret et al., *Plant J.* 16:651-659 (1998); Waterhouse et al., *Proc. Natl. Acad. Sci. USA* 95:13959-13964 (1998); Waterhouse et al., *Trends Plant Sci.* 4:452-457 (1999); Baulcombe, *Curr. Opin. Plant Biol.* 2:109-113 (1999)) and the fungus *Neurospora* ("quelling"; Cogoni et al., *EMBO J.* 15:3153-3163 (1996); Cogoni et al., *Nature* 399:166-169 (1999); Cogoni et al., *Science* 286:2342-2344 (1999)).

Recently, a number of publications have disclosed that RNAi can be observed in mammalian cells. Microinjection of dsRNA into mouse oocytes or early embryos results in specific inhibition of activity of both maternally and zygotically expressed proteins (Svoboda et al., *Development (Cambridge, U.K.)* 127:4147-4156 (2000); Wianny et al., *Nat. Cell Biol.* 2:70-75 (2000); PCT International Publication WO 01/36646)). PCT International Publication WO 01/36646, Elbashir et al. (*Nature* 411, 494-498 (2001)), Caplen et al. (*Proc. Natl. Acad. Sci. USA* 98:9742-9747 (2001)), and PCT International Publication WO 02/44321 disclose gene-specific silencing in cultured mammalian cells mediated by 21-25 nucleotide small interfering RNA (siRNA) duplexes (hereafter, the terms "dsRNA" and "siRNA" will be used interchangeably). In the same cultured cell systems, transfection of longer stretches of dsRNA yielded considerable non-specific silencing. PCT International Publication WO 01/75164 discloses RNA interference in human tissue cultures using 21-nt siRNA duplexes.

Several preliminary publications have suggested that RNAi can also be observed *in vivo* in adult mice and rats (Pachuk et al., *Keystone Symposia 2002*, Abstract 217, p. 115 (2002); Lewis et al., *Keystone Symposia 2002*, Abstract 312, p. 119 (2002); Rost et al., *Keystone Symposia 2002*, Abstract 323, p. 122 (2002)). More detailed results are reported in McCaffrey et al., *Nature* 418:38-39 (2002).

The recent discovery that 21-nucleotide RNA duplexes mediate RNAi in cultured mammalian cells distinguishes RNAi from the sequence-nonspecific responses of mammalian cells to long dsRNA. For example, most somatic mammalian cells undergo apoptosis when exposed to the concentrations of dsRNA that induce RNAi in invertebrate cells. The 21-nucleotide RNA duplexes are too short to elicit sequence-nonspecific responses like apoptosis, yet they efficiently intitiate RNAi. Furthermore, while exposure of mammals to dsRNAs greater than 30 basepairs (bp) in length induces an antiviral interferon response that globally represses mRNA translation (Kumar et al., *Microbiol. Mol. Biol. Rev.* 62:1415-1434 (1998); Stark et al., *Annu. Rev. Biochem.* 67:227-264 (1998)), introduction of shorter dsRNAs into mammalian cells leads to sequence-specific mRNA degradation without activating the interferon response (Caplen et al., *Proc. Natl. Acad. Sci. USA* 98:9742-9747 (2001)).

Matzke et al. (*Science* 293:1080-1083 (2001)), R.W. Carthew (*Curr. Opin. Cell Biol.* 13:244-248 (2001)), and Zamore (*Nature Structural Biol.* 8:746-750 (2001)) summarize recent developments in the understanding of the biochemistry of RNAi. Current models of RNAi include both an initiation step and an effector step (Hammond et al. (*Nature Rev. Gen.* 2:110-119 (2001))). In the initiation step, introduced dsRNA is digested into 21-23 nucleotide "guide RNAs," also referred to as siRNAs or "short interfering RNAs" (Hammond et al., *Nature Rev. Gen.* 2:110-119 (2001); P.A. Sharp, *Genes & Dev.* 15:485-490 (2001)). siRNAs appear to be produced when a nuclease complex, which recognizes the 3' ends of dsRNA, cleaves dsRNA introduced directly, or via a transgene or virus, approximately 22 nucleotides from the 3' end. Thereafter, successive cleavage reactions, catalyzed either by one complex or several complexes, degrade the RNA to 19-20 basepair duplexes, i.e., siRNAs, each having 2-nucleotide 3' overhangs. Since RNase III-type endonucleases cleave dsRNA to produce dsRNA fragments with 2-nucleotide 3' tails, an RNase III-like activity, termed "Dicer," appears to be involved in the RNAi mechanism. In view of the potency of RNAi in some organisms, it has been proposed that siRNAs are replicated by an RNA-dependent RNA polymerase (Hammond et al., *Nature Rev. Gen.* 2:110-119 (2001); P.A. Sharp, *Genes & Dev.* 15:485-490 (2001)). Interestingly, proposed models for RNAi suggest that the antisense strand of dsRNA determines mRNA target specificity (Yang et al., *Curr. Biol.* 10:1191-1200 (2000); R.W. Carthew (*Curr. Opin. Cell Biol.* 13:244-248 (2001)).

Recently, Sijen et al. (*Cell* 107:465-476 (2001)) and Lipardi et al. (*Cell* 107:297-307 (2001)) reported that siRNAs are amplified by an RNA-dependent RNA polymerase during RNA silencing.

Hepatitis C Virus

Besides infecting humans, HCV has been reported to infect hosts such as chimpanzees and tupias. Infection by the hepatitis C virus (HCV) is a compelling human medical problem, and HCV is now recognized as the causative agent for most cases of non-A, non-B hepatitis.

The HCV is thought to infect chronically 3% of the world's population (Alberti et al., *J. Hepatology* 31:(Suppl. 1) 17-24 (1999)). In the United States alone, the infection rate is 1.8% or 3.9 million people (M.J. Alter, *J. Hepatology* 31:(Suppl. 1) 88-91 (1999)). Of all patients infected, over 70% develop a chronic infection that is believed to be a major cause of cirrhosis and hepatocellular carcinoma (D. Lavanchy, *J. Viral Hepatitis* 6:35-47 (1999)).

The replication of the HCV relies on the viral genome, which encodes a polyprotein of 3010-3033 amino acids (Choo et al., *Proc. Natl. Acad. Sci. USA* 88:2451-2455 (1991); Kato et al., *Proc. Natl. Acad. Sci. USA* 87:9524-9528 (1990); Takamizawa et al., *J. Virol.* 65:1105-1113 (1991)). The HCV nonstructural (NS) proteins are presumed to provide the essential catalytic machinery for viral replication. The NS proteins are derived by proteolytic cleavage of the polyprotein (Bartenschlager et al., *J. Virol.* 67:3835-3844 (1993); Grakoui et al., *J. Virol.* 67:2832-2843 (1993); Grakoui et al., *J. Virol.* 67:1385-1395 (1993); Tomei et al., *J. Virol.* 67: 4017-4026 (1993)). In fact, the first 181 amino acids of NS3 (residues 1027-1207 of the viral polyprotein) have been shown to contain the serine protease domain of NS3 that processes all four downstream sites of the HCV polyprotein (Lin et al., *J. Virol.* 68:8147-8157 (1994)).

At present, there are no anti-HCV therapies except interferon-α, interferon-α/ribavirin combination, and more recently, pegylated inteferon-α. However, the sustained response rates for the interferon-α therapies and interferon-α/ribavirin tend to be low (<50%), and the side effects exhibited by the therapies tend to be significant and severe, including fatigue, depression, and anemia (M.A. Walker, *DDT* 4:518-529 (1999); Moradpour et al., *Eur. J. Gastroenterol. Hepatol.* 11:1199-1202 (1999); Janssen

et al., *J. Hepatol.* 21:241-243 (1994); Renault et al., *Seminars in Liver Disease* 9:273-277, (1989); G. Cowley, *Newsweek*, April 22, 2002, pp. 46-53). Furthermore, the interferon therapies induce long term remission in only a fraction (~25%) of cases (O. Weiland, *FEMS Microbiol. Rev.* 14:279-288 (1994)). The aforesaid problems with the interferon-α therapies have led to the development and clinical study of pegylated derivatized interferon-α compounds as improved anti-HCV therapeutics.

There exists a need in the art for compositions, including oligonucleotide therapeutics, and methods employing the same, to prevent or treat HCV infections in humans, including inhibiting infection, replication, and/or pathogenesis due to HCV, with minimal or no adverse side effects.

SUMMARY OF THE INVENTION

Accordingly, in view of the need in the art for effective therapies to treat or prevent HCV infections in humans, the present inventors have provided a number of polynucleotide compounds, formulations, and methods for inhibiting HCV infection, replication, and/or pathogenesis by various mechanisms, including by inhibiting the expression of HCV genes *in vivo*, as a means of treating or preventing such infections in human and animal patients. Therefore, the present invention is applicable in both human and veterinary medicine.

Thus, in a first aspect, the present invention provides an isolated double stranded RNA oligonucleotide about 19 to about 25 ribonucleotides in length,

wherein one strand of said isolated double stranded RNA oligonucleotide comprises the same nucleotide sequence as about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of a region of a hepatitis C virus target RNA polynucleotide sequence required for hepatitis C virus infection, replication, or pathogenesis *in vitro* or *in vivo* in a host cell,

wherein said isolated double stranded RNA oligonucleotide causes inhibition of infection, replication, or pathogenesis of said hepatitis C virus *in vitro* or *in vivo* when introduced into a host cell containing said hepatitis C virus, and

wherein said isolated double stranded RNA oligonucleotide exhibits an IC_{50} in the range of from about 0.0001 nM to about 1 μM in an *in vitro* assay for at least one step in infection, replication, or pathogenesis of said hepatitis C virus, or

a functionally equivalent variant of said isolated double stranded RNA oligonucleotide.

The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof can further comprise two overhanging 2'-deoxythymidine residues or two overhanging uridine residues at the 3' terminus of each strand of said isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof.

In another aspect, the present invention provides a composition, comprising an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof as disclosed above, and a buffer, carrier, diluent, or excipient.

In another aspect, the present invention provides a pharmaceutical composition, comprising an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof as disclosed above, and a pharmaceutically acceptable buffer, carrier, diluent, or excipient.

In another aspect, the present invention provides the use of an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof as disclosed above to prepare a medicament for the prevention or treatment of hepatitis C virus infection.

In another aspect, the present invention provides a method of inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence in a host cell, comprising:

introducing into said host cell an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof as disclosed above in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence in said host cell.

In yet another aspect, the present invention provides a method of inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence.

In another aspect, the present invention provides a method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell two or more isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said two or more isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

In another aspect, the present invention provides a method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in a different region in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

In another aspect, the present invention provides a method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein at least one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of at least one of said two or more hepatitis C virus target RNA polynucleotide sequences, and

at least two of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprise nucleotide sequences homologous to the nucleotide sequences of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of at least one other of said two or more hepatitis C virus target RNA polynucleotide sequences.

In any of the foregoing methods, inhibition of the function of said hepatitis C virus target RNA polynucleotide sequences can result in inhibition of hepatitis C virus infection, replication, or pathogenesis in said host cell. Furthermore, in any of the foregoing methods, each one of said isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof can be introduced into said host cell in an amount effective to completely inhibit the function of the hepatitis C virus target RNA polynucleotide sequence to which it corresponds, or to completely inhibit infection, replication, or pathogenesis of said hepatitis C virus in said host cell.

In yet another aspect, the present invention provides a method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof as disclosed above in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

In another aspect, the present invention provides a method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

wherein each one of said isolated multiple double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

In another aspect, the present invention provides a method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient two or more isolated double stranded RNA oligonucleotides, two or more functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said two or more isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

In still another aspect, the present invention provides a method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, multiple functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

In another aspect, the present invention provides a method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, multiple functionally equivalent variants thereof, or mixtures thereof, as disclosed above in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein at least one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of at least one of said two or more hepatitis C virus target RNA polynucleotide sequences, and

at least two of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprise nucleotide sequences homologous to the nucleotide sequences of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of at least one other of said two or more hepatitis C virus target RNA polynucleotide sequences.

In any of these methods, each one of said isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof can be administered to said human patient in an amount sufficient to completely inhibit the function of said hepatitis C virus target RNA polynucleotide sequence(s), or to completely inhibit infection, replication, or pathogenesis of said hepatitis C virus. Preferably, said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof comprise(s) two overhanging 2'-deoxythymidine residues or two overhanging uridine residues at the 3' terminus of each strand.

In still another aspect, the present invention provides a cell containing one or more isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof as disclosed above.

And in yet another aspect, the present invention provides a kit or pharmaceutical pack comprising reagents for inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence required for infection, replication, or pathogenesis of said virus in a host cell, comprising:

a DNA template nucleotide sequence of about 19 to about 25 nucleotides in length also comprising two different promoters selected from the group consisting of a T7 promoter, a T3 promoter, and an SP6 promoter, wherein each promoter is operably linked to said DNA template nucleotide sequence such that two complementary single stranded RNAs are transcribed from said DNA template nucleotide sequence, and wherein one complementary single stranded RNA of said two complementary single stranded RNA molecules comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence;

a plurality of primers for amplification of said DNA template nucleotide sequence;

nucleotide triphosphates for forming RNA;

at least two RNA polymerases, each capable of binding to a promoter on said DNA template nucleotide sequence and causing transcription of said nucleotide sequence to which the promoter is operably linked;

a purification column for purifying single stranded RNA;

buffer for annealing single stranded RNAs to yield double stranded RNA; and

RNAse A or RNAse T for purifying double stranded RNA,

wherein said DNA template nucleotide sequence encodes a double stranded RNA oligonucleotide or functionally equivalent variant as disclosed above.

Further scope of the applicability of the present invention will become apparent from the detailed description provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the present invention, are given by way of illustration only since various changes and

modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

The above and other aspects, features, and advantages of the present invention will be better understood from the following detailed description taken in conjunction with the accompanying drawing, all of which are given by way of illustration only and are not limitative of the present invention, in which:

Figure 1 shows the effect of single ribonucleotide mismatches introduced at every successive position in the active anti-HCV dsRNA LZ129 on the level of replicon RNA replication in Huh7 cells compared to that resulting from use of unmodified LZ129. Data are presented as mean ± standard error of the mean. The data show that single successive mismatches are well tolerated at the 5' and 3' ends of the dsRNA.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references, including patent documents, cited herein are herein incorporated by reference in their entirety.

Nature and Advantages of the Present Invention

The present invention is directed to the introduction of short dsRNAs, also referred to as siRNAs, into mammalian cells for the treatment or prevention of disease symptoms, conditions, or disorders caused by HCV. Use of dsRNAs for treating or preventing infection caused by HCV possesses a number of advantages. RNAi is a fundamental and highly conserved mechanism in many organisms. It is known that mammalian cells can respond to extracellular dsRNA, and may therefore possess a transport mechanism for dsRNA (Asher et al., *Nature* 223:715-717 (1969)). The method is sequence-specific, simple, fast, and can cause prolonged degradation of mRNAs

produced by the homologous gene (Fire et al., *Nature* 391:806-811 (1998); Ngo et al., *Proc. Natl. Acad. Sci. USA* 95:14687-14692 (1998); Yang et al., *Curr. Biol.* 10:1191-1200 (2000)). Assuming that the presently disclosed dsRNAs act through an RNAi mechanism, the use of approximately 18-25 nucleotide (nt) dsRNAs may provide optimal specificity for a homology-based searching mechanism. dsRNAs can be quickly analyzed by high-throughput screening in appropriate assay systems. Substoichiometric amounts of dsRNA can drastically decrease the levels of a great excess of homologous mRNA within a short time: individual 21-23 nucleotide RNAs may target multiple mRNAs for destruction (Fire et al., *Nature* 391:806-811 (1998); Kennerdell et al., *Cell* 95:1017-1026 (1998); Zamore et al., *Cell* 101:25-33 (2000); Yang et al., *Curr. Biol.* 10:1191-1200 (2000); Parrish et al., *Mol. Cell* 6:1077-1087 (2000)). An RNAi effect can persist for many rounds of cell division and growth even though the intitial dsRNA pool becomes diluted. Recent reports indicate that RNAi has an amplification mechanism, either via catalysis, synthesis, or possibly both (R.W. Carthew, *Curr. Opin. Cell Biol.* 13:244-248 (2001); Sijen et al. (*Cell* 107:465-476 (2001); Lipardi et al. (*Cell* 107:297-307 (2001)). RNAi has the ability to cross cell boundaries (Fire et al., *Nature* 391:806-811 (1998)). Post-transcriptional gene silencing by RNA interference can be simply induced by introducing *in vitro*-synthesized dsRNA into an organism. Antisense techniques, where a 1:1 hybridization of endogenous mRNA with antisense RNA is necessary, often fail to exhibit full gene silencing. Caplen et al. (*Proc. Natl. Acad. Sci. USA* 98:9742-9747 (2001)) observed that interference by short dsRNAs in cell lines from humans and mice was superior to the inhibition of gene expression mediated by single-stranded antisense oligonucleotides. These authors also noted that siRNAs seem to be very stable, and thus may not require the extensive chemical modification that single stranded RNA antisense oligonucleotides require to enhance their *in vivo* half-life. Catalytic amounts of dsRNA can promote complete degradation of homologous RNA. Elbashir et al. (*Nature* 411:494-498 (2001)) have noted that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene-targeting experiments. Note also PCT International Publication WO 99/32619 in this regard. The method is sequence-specific, and avoids the extensive non-specific effects triggered by the use of long dsRNAs in mammalian cells, including induction of the interferon

response, as discussed above. While RNase III is known to degrade long dsRNAs, purified 21-23mer species are resistant to RNase (Yang et al., *Curr. Biol.* 10:1191-1200 (2000)). Thus, short dsRNAs may not be subject to extensive RNase degradation in host cells, and such *in vivo* stability makes them attractive as therapeutic agents. dsRNAs are not limited to *in vitro* use, or to specific sequence compositions, target RNAs, particular portions of target RNA, or a particular delivery method.

To treat or prevent diseases and their associated pathologies caused by various pathogens, one can select one or more target pathogen genes or RNA species, or other regions of the pathogen genome that are transcribed into RNA and which are required for initiation or maintenance of the disease/pathology. Homologous dsRNA can be introduced into a human or animal patient or subject using *in vitro, ex vivo*, or *in vivo* methods. In an *in vitro* method, the dsRNA is introduced into a cell, and the dsRNA-containing cell is then introduced into the patient. In an *ex vivo* method, cells of the patient are explanted, the dsRNA is introduced into the explanted cells, and the dsRNA-containing cells are implanted back into the patient. In an *in vivo* method, dsRNA is administered directly to the patient. The dsRNA can also be delivered to a cell using one or more vectors that encode the complementary RNAs (or self-complementary RNA), which are then transcribed inside the cell and annealed to yield the desired dsRNA.

In the methods of the present invention, the target RNA, target gene, or other target genomic polynucleotide region is that of HCV. Collectively, these will be referred to herein as "target polynucleotide sequences," and each individually as a "target polynucleotide sequence." Generally, these can be either DNA or RNA. A candidate HCV target polynucleotide sequence might, for example, cause immunosuppression of the host, be involved in replication or transmission of the virus, or maintenance of the infection.

Attenuation of HCV infection, multiplication, spread, pathogenesis, gene expression in a cell or patient, etc., can be quantified, and the amount of attenuation of gene expression, etc., can be determined and compared to that in a cell or patient not treated according to the present invention. Lower doses of dsRNA may result in inhibition in a smaller fraction of cells, or in partial inhibition in cells. In addition, attenuation of gene expression, etc., can be time-dependent: the longer the period of time since the administration of the dsRNA, the less gene expression, etc., may be attenuated.

In such case, an appropriate dsRNA(s) can be readministered to achieve the desired effect. Attenuation of HCV infection, multiplication, spread, pathogenesis, gene expression, etc., within an infected human or animal host may occur at the level of nucleic acid replication (i.e., production of new HCV genomic RNA), transcription (i.e., accumulation of RNA, for example, but not limited to, mRNA, of the targeted gene), or translation (i.e., production of the protein encoded by the targeted gene). For example, HCV genomic RNA, which is a polycistronic mRNA, or other RNA from the targeted gene can be detected using a hybridization probe having a nucleotide sequence outside the region selected for the inhibitory double-stranded RNA; translated polypeptide encoded by the target gene can be detected via Western blotting using an antibody raised against the polypeptide. It should be noted that the methods of the present invention are not limited to any particular mechanism of reducing or eliminating cellular RNA or protein activity, or production or accumulation of any RNA species, associated with HCV infection and pathology. Indeed, it may not yet be fully understood how the introduction of short dsRNAs into a cell causes attenuation of expression of a targeted gene or activity of a targeted RNA species, nor is it known whether single or multiple mechanisms are involved. While the dsRNAs disclosed herein may produce their therapeutic effects via the mechanism known as RNA interference or RNA silencing, other mechanisms may be involved, and the present inventors do not wish to be bound to any particular theory regarding the mechanism of action of the presently disclosed dsRNAs.

The attenuation of HCV gene expression or RNA function achieved by the methods of the present invention are specific for the targeted gene or RNA species ("target polynucleotide sequence"). In other words, the dsRNA inhibits target HCV genes, or the function of HCV RNAs, without manifest effects on other genes or RNA species of the host cell.

dsRNA compounds are particularly useful in interfering with the life cycle of pathogens, and in treating or preventing infections or deleterious physiological conditions associated therewith. The present invention therefore encompasses methods of inhibiting HCV replication in cells, and for treating or preventing HCV infections in patients using the present dsRNA compounds or pharmaceutical compositions, as well as kits and pharmaceutical packs therefor. According to the present invention, included as

pharmaceutical compositions for treating HCV infections are compositions comprising one or more dsRNAs, alone or in combination.

dsRNAs of the present invention are designed such that they are homologous to RNA target sequences ("target RNA polynucleotide sequences") present in HCV, and can be administered or applied to affected humans or animals in the form of pharmaceutically acceptable compositions or formulations. It should be noted that the present methods are also useful for identifying RNA or protein targets within HCV that are necessary for viral infection, replication, and/or pathogenesis, against which new drugs can be designed and tested for therapeutic effectiveness in treating symptoms, conditions, or disorders associated with HCV infection. Thus, the compositions and methods of the present invention have utility both *in vitro* and *in vivo*. However achieved, one aspect of the present invention is to provide novel isolated dsRNAs that inhibit the function of the selected target RNA polynucleotide sequence, or of the peptide, polypeptide, or protein encoded thereby, thus providing complete relief or amelioration of the disease state caused by HCV. Interestingly, the present compositions and methods harness intracellular molecular mechanisms to accomplish their therapeutic goals. In humans, and probably other mammals as well, owing to the small size of the dsRNAs employed herein, the desired therapeutic effect(s) can be achieved without requiring any stimulation of the general immune response associated with the use of long dsRNAs, as discussed earlier.

The present invention provides dsRNA oligonucleotides and methods of use thereof for preventing or treating infections caused by HCV, which has a positive strand RNA genome. The dsRNA oligonucleotides are homologous to target RNA polynucleotide sequences of HCV, including protein-coding sequences thereof; non-protein coding sequences thereof; non-protein-coding HCV target polynucleotide sequences that provide viral regulatory functions; target RNA polynucleotide sequences or regions of the HCV viral RNA genome *per se*; and to any other RNA species involved in HCV infection, replication, and/or pathogenesis in a host cell or organism. The methods of the present invention facilitate modulation, attenuation, or inhibition of HCV infection, replication, and/or pathogenesis processes, which include, but are not limited to, HCV gene expression or function, in an infected cell or patient. "Modulation," "attenuation," or "inhibition" of HCV infection, replication, and/or pathogenesis

processes, including HCV gene expression or function, by the dsRNAs disclosed herein can be partial or complete. Such modulation, attenuation, or inhibition of HCV infection, replication, and/or pathogenesis processes, including HCV gene expression or function, can manifest itself as a reduction in any of these parameters in an amount of about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or about 100% compared to the same parameter in the absence of dsRNA corresponding to a region of the HCV target gene or other target polynucleotide sequence, especially a target RNA polynucleotide sequence. More particularly, such modulation, attenuation, or inhibition can manifest itself as a reduction in any of the above-noted parameters in an amount of at least about 50%, more preferably at least about 51%, yet more preferably at least about 52%, yet more preferably at least about 53%, yet more preferably at least about 54%, yet more preferably at least about 55%, yet more preferably at least about 56%, yet more preferably at least about 57%, yet more preferably at least about 58%, yet more preferably at least about 59%, yet more preferably at least about 60%, yet more preferably at least about 61%, yet more preferably at least about 62%, yet more preferably at least about 63%, yet more preferably at least about 64%, yet more preferably at least about 65%, yet more preferably at least about 66%, yet more preferably at least about 67%, yet more preferably at least about 68%, yet more preferably at least about 69%, yet more preferably at least about 70%, yet more preferably at least about 71%, yet more preferably at least about 72%, yet more preferably at least about 73%, yet more preferably at least about 74%, yet more preferably at least about 75%, yet more preferably at least about 76%, yet more preferably at least about 77%, yet more preferably at least about 78%, yet more preferably at least about 79%, yet more preferably at least about 80%, yet more preferably at least about 81%, yet more preferably at least about 82%, yet more preferably at least about 83%, yet more preferably at least about 84%, yet more preferably at least about 85%, yet more preferably at least about 86%, yet more preferably at least about 87%, yet more preferably at least about 88%, yet more preferably at least about 89%, yet more preferably at least about 90%, yet more preferably at least about 91%, yet more preferably at least about 92%, yet more preferably at least about 93%, yet more preferably at least about 94%, yet more preferably at least about 95%, yet more preferably at least about 96%, yet more preferably at least about 97%, yet more preferably at least

about 98%, yet more preferably at least about 99%, and most preferably about 100%, or any range therein.

Mechanistically, gene function can be partially or completely inhibited by, for example, blocking transcription from the gene to mRNA, by degrading mRNA, or by blocking translation of the mRNA to yield the protein encoded by the gene, although it should be understood that the present invention is not limited to any particular mechanism of modulation, attenuation, or inhibition of HCV gene expression or infection, replication, and/or pathogenesis by dsRNA. As discussed above, the mechanism of inhibition of gene expression by dsRNA is still presently under intensive investigation, and may not be fully elucidated at this time. Therefore, it should be understood that the dsRNAs disclosed herein are considered to modulate, attenuate, or inhibit the normal function of a target RNA or DNA polynucleotide sequence in a cell, whatever that function is. Modulation, attenuation, or inhibition of HCV infection, replication, and/or pathogenesis processes, including HCV gene expression or function, is evidenced by a reduction or elimination, in a cell or in a patient, of the activity associated with the target RNA (or DNA) polynucleotide sequence or the protein encoded by an HCV gene. It should be noted that the dsRNAs encompassed by the present invention can act on target polynucleotide sequences including HCV genomic RNA *per se* by causing degradation thereof, resulting in prevention or amelioration of disease symptoms, conditions, or disorders associated with HCV infection.

Whether and to what extent HCV infection, replication, and/or pathogenesis processes, including HCV gene expression or function, are inhibited can be determined using methods known in the art. For example, in many cases, inhibition of gene function leads to a change in phenotype which is revealed by examination of the outward properties of the cell or organism, or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP),

beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracycline. Inhibition of HCV replication can be monitored in blood or serum by, for example, immunological methods, or by measurement of viral RNA levels.

Target Polynucleotide Sequences

Any gene being expressed in a cell, or any RNA molecule present in a cell that is required for HCV infection, replication, and/or pathogenesis, can be targeted for inhibition of function or degradation by dsRNAs as disclosed herein. Such genes and RNA molecules are referred to as "target polynucleotide sequences," and can be either DNA or RNA. A gene that is expressed in a cell can be one that is transcribed to yield an mRNA and, optionally, translated into a peptide, polypeptide, or protein. In the case of HCV, which has an RNA genome, such target polynucleotide sequences can be regions of the RNA genome itself. Other target genes can include those that are transcribed to produce RNA endproducts other than mRNAs. The targeted gene or polynucleotide region can be chromosomal, i.e., genomic, or extrachromosomal. It may be endogenous to the cell, or it may be foreign, i.e., a transgene or, as in the present case, one or more HCV genes. The foreign gene can be integrated into the host genome, or it can be present on an extrachromosomal genetic construct such as a plasmid, cosmid, or virus genome. Target genes or DNA or RNA polynucleotide sequences may thus be any sequence present in a mammalian cell (homologous sequences) or HCV (heterologous sequences) that performs a function involved in HCV infection, replication, and/or pathogenesis which is to be reduced or entirely inhibited. The target polynucleotide sequence may be a protein-coding sequence, i.e., a polynucleotide sequence (either DNA or RNA) that is transcribed or translated to produce a peptide, polypeptide, or protein that can be structural or non-structural. Alternatively, the target DNA or RNA polynucleotide sequence may be non-protein-coding, for example one having a regulatory function. Thus, in general, a target polynucleotide sequence can be that of an intracellular

or extracellular pathogen, or a host cell, required for pathogen infestation, infection, replication, and/or pathogenesis, whether or not transcribed and/or translated, including regulatory sequences.

The present inventors have recognized that dsRNAs effective in inhibiting HCV infection, replication, and/or pathogenesis *in vitro* or *in vivo* are not limited exclusively to dsRNAs corresponding to nucleotide sequences within HCV genomic RNA that encode a peptide, polypeptide, or protein; or that regulate replication, transcription, translation, or other processes involving HCV target RNA polynucleotide sequences, including the expression of peptides/polypeptides/proteins; or to polynucleotides comprising both a region that encodes a peptide, polypeptide, or protein and a region operably linked thereto that regulates expression. The present dsRNAs and methods of prevention and treatment also encompass the use of dsRNAs that correspond to target polynucleotide sequences within other RNA species, including untranslated RNA species such as the HCV 5' and 3' untranslated regions (UTRs) and negative sense RNA strand, required for HCV infection, replication, and/or pathogenesis. These RNA polynucleotide species include, but are not limited to, viral genomic RNA *per se* , including protein-coding (exon) and non-protein-coding regions therein; transfer RNAs; ribosomal RNAs; splicosomal RNAs; host cell mRNAs or other RNAs, including non-protein coding RNAs, required for infection/replication/pathogenesis; small RNAs, such as small (tiny) temporal RNAs, either pathogen or host cell, that may regulate genes involved in HCV infection, replication, and/or pathogenesis (see Hutvagner et al., *Science* 293:834-838 (2001) and Grishok et al., *Cell* 106:23-34 (2001) for a discussion of small temporal RNAs); regulatory RNAs; and any other RNAs involved in HCV infection, replication, and/or pathogenesis. For purposes of the present inventions, the RNA genome of HCV is considered to include the positive sense RNA strand as well as the negative sense RNA strand of HCV.

Persons of ordinary skill in the art will understand that messenger RNA includes not only the sequence information to encode a protein using the three letter genetic code, but also associated ribonucleotides that form regions such as the 5'-untranslated region, the 3'-untranslated region, and the 5' cap region, as well as ribonucleotides that form various secondary structures. Thus, dsRNA oligonucleotides may be formulated in accordance with this invention that are targeted wholly or in part to these associated

ribonucleotides as well as to the coding ribonucleotides. Thus, the compounds, compositions, and therapeutic methods disclosed herein are not limited exclusively to pathogens, including viruses, that possess DNA genomes, or which utilize DNA-RNA transcription or RNA-protein translation as a fundamental part of their metabolic or cellular control. Diverse organisms, including those having RNA genomes (such as HCV), are susceptible to the present methods, and control of these organisms can be achieved at levels other than, or in addition to, gene expression involving nucleic acid transcription and translation.

As used herein, the terms "mammal" or "mammalian" encompass their normal meaning as known in the art. While the present invention is applicable to human diseases, it is equally applicable to diseases of animals such as chimpanzees and tupias.

The cell harboring the target RNA polynucleotide sequence can be a primate cell, more particularly a human cell, more particularly a human liver cell, and even more particularly a human hepatocyte.

Double Stranded RNAs

The dsRNAs useful in the present invention can be formed from one or more strands of polymerized ribonucleotides. While dsRNA duplexes with blunted ends and 1-nucleotide (nt) 5' overhangs can be functional, preferred dsRNAs active as siRNAs have 5'-phosphate/3'-hydroxyl termini and 2-, 3-, 4-, or 5-nt 3'overhangs on each strand of the duplex, with 2-nt 3' overhangs being preferred (Caplen et al., *Proc. Natl. Acad. Sci. USA* 98:9742-9747; PCT International Publication WO 02/44321). Isolated dsRNAs of the present invention can have two 2'-deoxythymidine or two uridine residues at the 3' end of each strand of a dsRNA duplex. The absence of a 2-hydroxyl group significantly enhances the nuclease resistance of the overhang under physiological conditions. The use of dsRNA duplexes with identical 3' overhanging sequences may be preferred (PCT International Publication WO 02/44321).

dsRNA-associated gene-specific responses may, at some stage, involve the pairing of antisense RNA sequences derived from the siRNA with the endogenous sense RNA (Parrish et al., *Mol. Cell* 6:1077-1087 (2000)). These authors investigated the chemical and sequence requirements for RNAi in *Caenorhabditis elegans* using dsRNAs in the range from 26 to 81 nucleotides, 62 to 242 nucleotides, and 65 to 717 nucleotides. They demonstrated a requirement for double stranded character in the interfering RNA, i.e.,

that an effective dsRNA requires a sense/antisense duplex in the region of identity to the target polynucleotide sequence. These authors also demonstrated that absolute homology between dsRNA and the corresponding target RNA is not required. Mismatches between strands of dsRNA duplexes were also tolerated: such heteroduplexes were effective in RNA interference, with reduced effectiveness when compared to perfectly matched duplexes. No significant difference was observed as a function of which strand was more closely related to the RNAi target. Modification of the sugar-phosphate backbone in the dsRNA, i.e., replacement of phosphate residues with thiophosphate residues, was investigated. Interference activity was observed following incorporation of any single modified residue. Modifications of A, C, or G residues were compatible with full interference activity, while modified U caused some decrease in interference activity. dsRNAs with two modified bases exhibited substantial decreases in effectiveness in RNA interference. Modification of more than two residues greatly destabilized the dsRNAs *in vitro*, and the authors were not able to assay interference activities.

Modifications were also tested at the 2'- position of the nucleotide sugar. Modification of cytidine to deoxycytidine (or uracil to thymidine) on either the sense or the antisense strand of the dsRNA produced a substantial decrease in interference activity. Replacement of uracil with 2'-fluorouracil was compatible with RNAi activity, while substitution of uracil with 2'-aminouracil or 2'-aminocytidine resulted in a decrease in activity.

RNA:DNA hybrids were found to lack interference activity under the conditions studied. Furthermore, dsRNA activity was more sensitive to several modifications (uracil → 2'-aminouracil; cytidine → 2'-aminocytidine; uracil → thymine; and cytidine →2'-deoxycytidine) of the antisense strand than of the sense strand.

As to base modifications, 4-thiouracil and 5-bromouracil were compatible with interference. Inosine was also compatible, but produced a substantial decrease in interference activity. There was no detectable difference in effect when these substitutions were made in either of the two strands of the dsRNA duplex. In contrast, 5-iodouracil and 5-(3-aminoallyl)uracil were compatible with interference, albeit at reduced levels compared to unmodified dsRNA, and resulted in substantially greater negative effects on RNA interference when present in the antisense strand of the duplex. Greater reduction in RNA interference due to replacement of uracil with 5-(3-aminoallyl)uracil in

the antisense strand compared to the sense strand was observed in a number of different dsRNA segments. The authors speculated that large substituents at the 5-position of uracil may act by sterically blocking recognition or catalysis at a key step in RNAi.

Injection of uniformly ^{32}P-labeled dsRNA into *C. elegans* resulted in RNA interference. Autoradiography of RNAs extracted from injected animals followed by polyacrylamide gel electrophoresis revealed that a substantial fraction of the injected dsRNA persisted as high molecular weight material, and that the injected dsRNA appeared to be cleaved into a population of short RNA segments approximately 25 nucleotides long. A relatively small fraction of cleaved RNAs was observed, accounting for approximately 1% of the recovered radioactivity.

In summary, Parrish et al. noted that key features of RNAi as observed in *C. elegans* under the conditions they employed include the double stranded nature of the triggering RNA, the ability to target native or foreign mRNAs for degradation, and the effectiveness of a broad range of dsRNA sequences in this process. RNA duplexes in a wide range of lengths having a variety of nucleotide compositions were effective. There did not appear to be a specific requirement for any sequence motif either in the dsRNA or the target RNA, and there did not appear to be any requirement for A, U, or C residues in the targeted sequence. Chemical modifications that tended to reduce helical A form character, such as 2'-deoxy and 2'-amino substitutions for one of the four bases on one of the two trigger dsRNA strands, decreased the effectiveness of the dsRNA duplex. Modification of at least one-quarter of bases to a 2' fluoro group, which preserves A form structure, was compatible with dsRNA function in inducing RNAi. Large chemical substitutions at the 5- position of uracil were compatible with effective RNAi when present in the sense strand, but not the antisense strand, of the dsRNA. Various modifications to the backbone in the antisense strand preferentially blocked RNAi. The authors concluded that the two strands of the dsRNA duplex have distinct roles in the RNAi process, and suggested that the antisense strand could interact directly with the target RNA, while the sense strand participates indirectly in the RNAi process, perhaps by protecting the antisense strand from degradation and allowing recognition of the dsRNA molecule as an RNAi trigger molecule. Substitution of inosine for guanine and sequence divergence decreased dsRNA activity independent of which dsRNA strand was

modified. Finally, the authors noted that parameters of the RNAi gene silencing mechanism may vary among different biological systems.

It should be noted that, for the purposes of the present invention, bases or sugars within naturally occurring ribonucleotides may be modified, so that the term "ribonucleotide" as used herein encompasses not only naturally occurring ribonucleotides normally found within RNA, but also modified ribonucleotides or ribonucleotide analogs, or even deoxyribonucleotides. For example, any base within a ribonucleotide can be substituted with inosine.

When formed from only one strand, dsRNA can take the form of a self-complementary hairpin-type molecule that doubles back on itself to form a duplex. Thus, the dsRNAs of the present invention can be fully or only partially double-stranded. Specific inhibition of gene activity can be achieved by stable expression of dsRNA hairpins in transgenic lines (Hammond et al., *Nat. Rev. Genet.* 2:110-119 (2001); Matzke et al., *Curr. Opin. Genet. Dev.* 11:221-227 (2001); P.A. Sharp, *Genes Dev.* 15:485-490 (2001)). When formed from two strands, or a single strand that takes the form of a self-complementary hairpin-type molecule doubled back on itself to form a duplex, the two strands (or duplex-forming regions of a single strand) are complementary RNA strands that base pair in Watson-Crick fashion.

In some situations, it may be desirable to employ a dsRNA wherein the duplex-forming strands or regions are less than fully complementary, for example about 80% to about 99% complementary or any individual value within this range, since a lower degree of complementarity will permit these strands or regions to separate more easily, facilitating binding of the antisense strand or region to its target polynucleotide sequence. In such cases, it is preferred that the sense strand or region of the duplex be modified to lack exact complementarity to the antisense strand or region. For example, lowering the G and/or C content of the sense strand or region, or incorporating one or more mismatched ribonucleotides therein relative to the complementary position(s) in the antisense strand or region, can destabilize a dsRNA in a useful manner.

The pharmacological activity of dsRNA oligonucleotides, like other therapeutics, depends on a number of factors that influence the effective concentration of these agents at specific intracellular targets. One important factor for oligonucleotides is the stability of the nucleic acid species in the presence of nucleases. Unmodified, naturally-occurring

dsRNA oligonucleotides may not be optimal therapeutic agents due to potential susceptibility to rapid degradation by nucleases, and may therefore require modifications that provide resistance to nucleases, as well as satisfactory hybridization properties. The dsRNA can include modifications to either the phosphate sugar backbone, the sugar, or the nucleoside base. For example, the phosphodiester linkages of natural RNA may be modified to increase the nuclease stability of the resulting analog by including at least one of a nitrogen or sulfur heteroatom. Examples of such modifications include incorporation of methyl phosphonate, phosphorothioate, or phosphorodithioate linkages, and 2'-sugar modifications such as 2'-*O*- (2-methoxyethyl) ribose sugar units and others as disclosed in U.S. Patent Application Publication No. 2001/0016652A1 and in U.S. Patent No. 6,284,458. Phosphorothioate oligonucleotides are presently being used as antisense agents in human clinical trials for various disease states, including use as antiviral agents.

Likewise, bases may be modified to block the activity of adenosine deaminase. Further modifications, such as those disclosed in U.S. Patent Application Publication No. 2001/0027251A1, include those made to enhance the activity, cellular distribution, or cellular uptake of the dsRNA oligonucleotide. Other modifications that improve *in vivo* stability of RNA molecules are well known in the art as disclosed in the following patent documents, which list is only meant to be illustrative rather than limitative: U.S. Patents 6,284,458; 6,277,967; 6,242,589; 6,132,966; 6,022,962; 5,869,253; 5,837,855; 5,767,263; 5,686,599; 5,646,020; 5,631,359; and 5,610,054, and in PCT International Publications WO 01/16312; WO 00/75306; and WO 99/05094.

The present invention encompasses chimeric or mixed backbone dsRNA compounds. As used herein, the term "chimeric or mixed backbone dsRNA" refers to dsRNA compounds comprising nucleoside monomer subunits and containing at least two different internucleoside linkages. The mixed backbone dsRNA compounds of the present invention may contain a plurality of nucleoside monomer subunits that are joined together by more than one type of internucleoside linkages. At least one internucleoside linkage is a phosphodiester linkage, and at least one other linkage is a phosphorothioate, a phosphoramidate or a boranophosphate internucleoside linkage as disclosed in United States Patent Application Publication No. 2001/0016652A1. Other possible internucleotide linkages include methyl phosphate, methylphosphonate, alkylphosphonate, S-aryl phosphorothioate, acylphosphonate, phosphorofluoridate,

phosphorodithioate, selenophosphate, and (hydroxymethyl)phosphonate. It should be noted that some modifications to the phosphodiester backbone of the dsRNAs encompassed by the present invention can result in RNA duplex strands to which conventional 5'-3' or 3'-5' terminology is inapplicable, e.g., 5'-2' linkages. RNA duplex strands containing such 5'-2' or other unconventional backbone linkages are considered equivalents of conventional 5'-3' or 3'-5' strands for the purposes of the present invention. dsRNAs of the present invention may also comprise sugar mimetics such as carbocyclic sugars such as cyclobutyls; acyclic sugars; sugars having substituent groups at their 2' position; and sugars having substituents in place of one or more hydrogen atoms of the sugar, in place of the pentofuranosyl group. Other altered sugar moieties are disclosed in PCT International Publication WO 89/12060 and U.S. Patent Nos. 6,320,040 and 6,307,040.

dsRNAs of the present invention can be RNA/RNA hybrids or RNA/DNA hybrids. In the latter, a single nucleic acid strand can contain both RNA and DNA, or a duplex of two such single chains or portions thereof. In another embodiment, the duplex molecule can comprise an RNA single strand and a DNA single strand.

As used herein, the term "nucleoside" refers to a unit composed of a heterocyclic (usually nitrogen-containing) base and its sugar. The term "nucleotide" refers to a nucleoside having a phosphate group on its 3' or 5' sugar hydroxyl group. As used herein, the term "oligonucleotide" is intended to include both naturally occurring and non-naturally occurring ("synthetic") oligomers of linked nucleosides. Although such linkages are generally between the 3' carbon of one nucleoside and the 5' carbon of a second nucleoside, i.e., 3'-5' linkages, other linkages, such as 2'- 5' linkages, can be present.

Naturally occurring oligonucleotides are those that occur in nature, for example ribose and deoxyribose phosphodiester oligonucleotides having adenine, guanine, cytosine, thymine, and uracil bases. As used herein, non-naturally occurring oligonucleotides are oligonucleotides that contain modified sugar, internucleoside linkage, and/or base moieties. Such oligonucleotide analogs are typically structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic wild-type oligonucleotides. Thus, non-naturally occurring oligonucleotides include all such structures that function effectively to mimic the structure and/or function

of a desired dsRNA strand, for example, by hybridizing to a target, or causing the degradation thereof.

Representative heterocyclic nitrogenous bases include purines such as adenine and guanine, and pyrimidines such as cytosine, thymine, and uracil. Other natural and non-naturally occurring bases include deaza or aza purines and pyrimidines; pyrimidines having substituent groups at the 5- or 6- position; purines having altered or replacement substituent groups at the 2-, 6-, or 8- positions; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other alkyl derivatives of adenine and guanine; 2-propyl and other alkyl derivatives of adenine and guanine; 5-halo uracil and cytosine; 6-azo uracil, cytosine, and thymine; 5-uracil (pseudouracil); 4- thiouracil; 8-halo, oxa, amino, thiol, thioalkyl, hydroxyl, and other 8- substituted adenines and guanines; 5-trifluoromethyl and other 5-substituted uracils and cytosines; and 7-methylguanine. Specific non-limiting examples of natural and non-natural bases include 4-acetylcytidine; 5-(carboxyhydroxy-methyl)uridine; 2'-*O*-methylcytidine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyluridine; dihydrouridine; 2'-*O*-methylpseudouridine; beta, D-galactosylqueuosine; 2'-*O*-methylguanosine; inosine; N6-isopentenyladenosine; 1-methyladenosine; 1-methylpseudouridine; 1-methylguanosine; 1-methylinosine; 2,2-dimethylguanosine; 2-methyladenosine; 2-methylguanosine; 3-methylcytidine; 5-methylcytidine; N6-methyladenosine; 7-methylguanosine; 5-methylaminomethyl-uridine; 5-methoxyaminomethyl-2-thiouridine; beta, D-mannosylqueuosine; 5-methoxycarbonylmethyl-2-thiouridine; 5-methoxycarbonylmethyluridine; 5-methoxyuridine; 2-methylthio-N6-isopentenyladenosine; N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine; N-((9-beta-D-ribofuranosylpurine-6-yl)N-methylcarbamoyl)threonine; uridine-5-oxyacetic acid-methylester; uridine-5-oxyacetic acid; wybutoxosine; pseudouridine; queuosine; 5-methyl-2-thiouridine; 2-thiocytidine; 5-methyl-2-thiouridine; 2-thiouridine; 4-thiouridine; 5-methyluridine; N-((9-beta-D-ribofuranosylpurine-6-yl)-carbamoyl)threonine; 2'-*O*-methyl-5-methyluridine; 2'-*O*-methyl-5-methyluridine; 2'-*O* methyluridine; wybutosine; and 3-(3-amino-3-carboxy-propyl)uridine ((acp3)u).

Further naturally and non-naturally occurring bases include those disclosed in U.S. Patent No. 3,687,808 (Merigan, et al.); PCT International Publication WO 89/12060; chapter 15 of Sanghvi, *Antisense Research and Application*, S. T. Crooke and B. Lebleu,

eds., CRC Press, 1993; Englisch et al., *Angewandte Chemie*, International Edition, 30: 613-722 (note especially pages 622 and 623) (1991); the *Concise Encyclopedia of Polymer Science and Engineering*, J. I. Kroschwitz Ed., John Wiley & Sons, pp. 858-859 (1990); and in Cook, *Anti-Cancer Drug Design* 6:585-607 (1991). The term "nitrogenous base or nucleosidic base" is further intended to include heterocyclic compounds that can serve as like nucleosidic bases, including certain "universal bases" that are not nucleosidic bases in the most classical sense, but that serve as nucleosidic bases, for example 3- nitropyrrole. Hypoxanthine, another universal base, is present in inosine (hypoxanthine riboside). Universal bases can be used at nucleotide positions in dsRNAs of the present invention that correspond to nucleotide positions in target RNAs that are variable among strains of HCV as such bases are capable of Watson-Crick base pairing with A, C, G, or T.

Oligonucleotides and their analogs can be synthesized to possess customized properties that can be tailored for desired uses. A number of chemical modifications have been introduced into oligomeric compounds to increase their usefulness as therapeutic entities. Such modifications include those designed to increase binding to a target strand (i.e., increase their melting temperature, Tm), to assist in identification of the oligonucleotide or an oligonucleotide-target complex, to increase cell penetration, to stabilize against nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotides and their analogs, to provide a mode of disruption (terminating event) once sequence-specifically bound to a target, and to improve the pharmacokinetic properties of the oligonucleotide.

Thus, for the purposes of the present invention, the terms "dsRNA," "dsRNA oligonucleotide," or "siRNA" and the like as used herein encompass any of the modifications discussed herein.

The nucleotide sequence of each of the anti-HCV dsRNAs disclosed herein is defined by the nucleotide sequence of the corresponding region of its target gene, other target RNA polynucleotide sequence, or HCV genomic region ("target RNA polynucleotide sequence"). For example, the 5'-3' nucleotide sequences of the dsRNA oligonucleotide pairs disclosed herein that target HCV genomic regions correspond to discrete, contiguous 5'-3' sequences of similar length within the positive strand RNA genome of HCV.

The present dsRNAs thus contain a nucleotide sequence that is identical or essentially identical in nucleotide sequence to at least a region of the target gene, target RNA polynucleotide sequence, or target RNA genomic region, but in addition contain two 2'-deoxythymidine or two uridine nucleotides at the 3' terminal end of each strand of each RNA oligonucleotide duplex. Interestingly, Elbashir et al. (*Genes & Dev.* 15:188-200 (2001)) have noted that the most active synthetic small interfering RNAs (siRNAs) directing RNAi *in vitro* contain two-nucleotide, 3' overhanging ends. Preferably, the dsRNA contains a strand (if it is a duplex) or region (if it is a self-complementary single stranded RNA) comprising a nucleotide sequence that is completely identical in nucleotide sequence to a region of the target polynucleotide sequence. The dsRNAs of the present invention can be about 14 to about 25 nucleotides in length, and a strand or region thereof should correspond in nucleotide sequence to about 14 to about 25 contiguous corresponding nucleotides in the target polynucleotide sequence. It should be understood that in comparing an RNA sequence to a DNA sequence, an "identical" RNA sequence will contain ribonucleotides where the DNA sequence contains deoxyribonucleotides, and further that the RNA sequence will contain a uracil at positions where the DNA sequence contains thymidine. More preferably, the dsRNA that is completely identical in nucleotide sequence to a region of the target polynucleotide sequence does not contain any additional nucleotides, except for the two 2'-deoxy-thymidine or two uridine nucleotides at the 3' terminal end of each strand of the RNA oligonucleotide duplex. The region of the target gene, RNA species, or genomic region to which the dsRNA sequence is essentially or completely sequence identical is preferably a sequence that is unique to the genome of HCV. The siRNA duplexes specifically exemplified below are composed of 21 nucleotide sense and 21 nucleotide antisense strands, paired so as to have a 19 nucleotide duplex region and a two nucleotide 2'-deoxythymidine or uridine overhang at each 3'-terminus.

A dsRNA useful in the present invention that is identical or "essentially identical" to at least a portion of an HCV target gene, an HCV RNA polynucleotide sequence required for viral infection, replication, and/or pathogenesis, or an HCV RNA genomic sequence or region, collectively referred to as a "target polynucleotide sequence," is a dsRNA wherein one of the two complementary strands (or, in the case of a self-

complementary RNA, one of the two self-complementary portions) is either completely identical to the sequence of a portion of the target polynucleotide sequence (100% sequence identity, sometimes also referred to herein as "sequence homology," "homology," or the like), or contains one or more insertions, deletions, or single point mutations compared to the nucleotide sequence of a portion of the target polynucleotide sequence that does not adversely affect the ability of the dsRNA to inhibit the function of the target polynucleotide sequence. The present invention thus possesses the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, serotype differences, or evolutionary divergence in HCV strains of interest. Other means of overcoming sequence variations in HCV target polynucleotide sequences besides introducing mismatches into dsRNA oligonucleotides include using shorter dsRNAs, e.g., 14-18 nt in length; introducing universal bases such as inosine into dsRNA oligonucleotides; using a combination of different dsRNA oligonucleotides that target multiple regions of single target polynucleotide sequences, multiple target polynucleotide sequences, or combinations thereof; or combinations of all these techniques. Alternatively, a dsRNA that is identical or "essentially identical" to at least a portion of the target polynucleotide sequence can be functionally a dsRNA wherein one of the two complementary strands (or, in the case of a self-complementary RNA, one of the two self-complementary portions) is capable of hybridizing with a portion of the target polynucleotide sequence, for example under conditions including 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours, followed by washing, or the equivalent thereof.

Functionally equivalent variants of the presently disclosed dsRNAs effective in silencing HCV gene expression or otherwise modulating, attenuating, or inhibiting HCV infection, multiplication, and/or pathogenesis that are encompassed by the present invention can be identified *in silico* by comparing their structural similarity, or sequence homology (sequence identity), to the presently disclosed dsRNAs. A dsRNA possessing about 25% or greater sequence identity, more preferably about 30% or greater sequence identity, more preferably about 35% or greater sequence identity, more preferably about 40% or greater sequence identity, more preferably about 45% or greater sequence identity, more preferably about 50% or greater sequence identity, more preferably about 55% or greater sequence identity, more preferably about 60% or greater sequence

identity, more preferably about 65% or greater sequence identity, more preferably about 70% or greater sequence identity, more preferably about 75% or greater sequence identity, more preferably about 80% or greater sequence identity, more preferably about 85% or greater sequence identity, more preferably about 90% or greater sequence identity, more preferably about 95% or greater sequence identity, more preferably about 98% or greater sequence identity, more preferably about 99% or greater sequence identity, especially 75%-95% or greater sequence identity, even more especially 85%-95% or greater sequence identity, even more especially 90%-95% or greater sequence identity, and even more especially 95%-100% sequence identity to any of the presently disclosed dsRNAs or fragments thereof is considered a functional variant encompassed by the present invention if it is capable of inhibiting the expression of an HCV target gene, or otherwise inhibiting the function of an HCV target polynucleotide sequence required for viral infection, multiplication, and/or pathogenesis containing a corresponding ribonucleotide sequence to substantially the same extent, i.e., within about ±10-25%, as the corresponding dsRNA disclosed herein. It should be noted that dsRNA sequences containing insertions, deletions, and single point mutations relative to the target polynucleotide RNA or DNA sequence have been found to be effective for RNAi inhibition (PCT International Publication WO 99/32619). Note also Parrish et al., *Mol. Cell* 6:1077-1087 (2000) in this regard. As shown in Example 2, below, this is true in the case of the present anti-HCV dsRNAs as well.

To determine sequence homology (sequence identity), a variety of different mathematical algorithms can be used. Identity can be readily calculated using, for example, the methods and algorithms disclosed in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York (1993); *Computer Analysis of Sequence Data, Part 1*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York (1991). There exist a number of different methods to measure identity between two polynucleotide sequences, and the term is well known to skilled artisans (*Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton

Press, New York, (1991); and Carillo, H., and Lipman, D., *SIAM J. Applied Math.* 48:1073 (1988).

Methods commonly employed to determine identity between sequences include, but are not limited to, those disclosed in Carillo, H., and Lipman, D., *SIAM J Applied Math.* 48:1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity are codified in computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux et al., *Nucleic Acids Research* 12(l):387 (1984)), BLASTP, BLASTN, and FASTA (Atschul et al., *J. Mol. Biol.* 215:403 (1990)). Another software package well known in the art for carrying out this procedure is the CLUSTAL program, which compares the sequences of two polynucleotides and finds the optimal alignment by inserting spaces in either sequence as appropriate.

The identity for an optimal alignment can also be calculated using a software package such as BLASTx. This program aligns the largest stretch of similar sequence and assigns a value to the fit. For any one pattern comparison, several regions of similarity may be found, each having a different score. One skilled in the art will appreciate that two polynucleotides of different lengths may be compared over the entire length of the longer fragment. Alternatively, small regions may be compared. Normally, sequences of the same length are compared for a useful comparison to be made. Searches for sequence similarities (as well as sequence uniqueness to insure specificity of dsRNA action) in databases enable the designing of dsRNAs for use in the methods of the present invention.

Selection of the necessary level of homology or sequence identity, selection of the defaults for the program employed, and selection of the program employed to calculate homology are within the ordinary skill of the art.

Although any sequence algorithm can be used to determine sequence homology (identity) in order to identify or design functionally equivalent variants of the presently disclosed dsRNAs, the present invention defines functional variants with reference to the Smith-Waterman algorithm (Smith and Waterman, *J. Mol. Biol.*, 147:195-197 (1981); Pearson, *Genomics*, 11:635-650 (1991)), where a dsRNA sequence as disclosed herein or a fragment thereof is used as the reference sequence to define the percentage of homology

of polynucleotide homologues over its length. The choice of parameter values for matches, mismatches, and inserts or deletions is arbitrary, although some parameter values have been found to yield more biologically realistic results than others. One preferred set of parameter values for the Smith-Waterman algorithm is set forth in the "maximum similarity segments" approach, which uses values of 1 for a matched residue and –1/3 for a mismatched residue (a residue being a either a single nucleotide or single amino acid) (Waterman, *Bulletin of Mathematical Biology* 46:473-500 (1984)). Insertions and deletions x, are weighted as

$$x_k = 1 + k/3,$$

where k is the number of residues in a given insert or deletion (*Id.*).

Preferred functionally equivalent variant dsRNA oligonucleotides of the present invention are those having at least about 50% sequence identity, more preferably at least about 55% sequence identity, more preferably at least about 60% sequence identity, more preferably at least about 65% sequence identity, more preferably at least about 70 % sequence identity, and even more preferably about, or at least about, 75% sequence identity to a dsRNA disclosed herein, or a fragment thereof, using the Smith-Waterman algorithm. More preferred functionally equivalent variant dsRNA oligonucleotides have at least about 80% sequence identity, more preferably at least about 85% sequence identity, more preferably at least about 90% sequence identity, more preferably at least about 95% sequence identity, more preferably at least, or at least about, 98% sequence identity, and even more preferably at least about 99% sequence identity to a dsRNA or fragment thereof disclosed herein.

In more specific embodiments, functionally equivalent variant dsRNA oligonucleotides of the present invention comprise dsRNAs having at least about 50% sequence identity, preferably at least about 51% sequence identity, more preferably at least about 52% sequence identity, yet more preferably at least about 53% sequence identity, yet more preferably at least about 54% sequence identity, yet more preferably at least about 55% sequence identity, yet more preferably at least about 56% sequence identity, yet more preferably at least about 57% sequence identity, yet more preferably at least about 58% sequence identity, yet more preferably at least about 59% sequence identity, yet more preferably at least about 60% sequence identity, yet more preferably at least about 61% sequence identity, yet more preferably at least about 62% sequence

identity, yet more preferably at least about 63% sequence identity, yet more preferably at least about 64% sequence identity, yet more preferably at least about 65% sequence identity, yet more preferably at least about 66% sequence identity, yet more preferably at least about 67% sequence identity, yet more preferably at least about 68% sequence identity, yet more preferably at least about 69% sequence identity, yet more preferably at least about 70% sequence identity, yet more preferably at least about 71% sequence identity, yet more preferably at least about 72% sequence identity, yet more preferably at least about 73% sequence identity, yet more preferably at least about 74% sequence identity, yet more preferably at least about 75% sequence identity, yet more preferably at least about 76% sequence identity, yet more preferably at least about 77% sequence identity, yet more preferably at least about 78% sequence identity, yet more preferably at least about 79% sequence identity, yet more preferably at least about 80% sequence identity, yet more preferably at least about 81% sequence identity, yet more preferably at least about 82% sequence identity, yet more preferably at least about 83% sequence identity, yet more preferably at least about 84% sequence identity, yet more preferably at least about 85% sequence identity, yet more preferably at least about 86% sequence identity, yet more preferably at least about 87% sequence identity, yet more preferably at least about 88% sequence identity, yet more preferably at least about 89% sequence identity, yet more preferably at least about 90% sequence identity, yet more preferably at least about 91% sequence identity, yet more preferably at least about 92% sequence identity, yet more preferably at least about 93% sequence identity, yet more preferably at least about 94% sequence identity, yet more preferably at least about 95% sequence identity, yet more preferably at least about 96% sequence identity, yet more preferably at least about 97% sequence identity, yet more preferably at least about 98% sequence identity, yet more preferably at least about 99% sequence identity, or any range therein, to a dsRNA or fragment thereof disclosed herein.

The dsRNA ribonucleotide sequence that is essentially or completely identical to at least a corresponding contiguous portion of the target polynucleotide sequence preferably has a length (excluding 5' or 3' overhangs) in the range of from about 10 nucleotides to about 100 nucleotides, more preferably from about 10 nucleotides to about 50 nucleotides, more preferably from about 10 nucleotides to about 40 nucleotides, more preferably from about 10 nucleotides to about 30 nucleotides, more preferably from about

10 nucleotides to about 25 nucleotides, from about 10 nucleotides to about 20 nucleotides, from about 14 nucleotides to about 25 nucleotides, from about 14 nucleotides to about 20 nucleotides, from about 17 nucleotides to about 25 nucleotides, from about 18 nucleotides to about 25 nucleotides, or from about 19 nucleotides to about 25 nucleotides, i.e., 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, or 30 nucleotides, or any range therein. It should be understood that the length of the dsRNA, the degree of homology necessary to affect HCV gene expression, RNA function, or viral infection, replication, and/or pathogenesis, and the most effective dosage for any particular dsRNA disclosed herein, can be optimized for each particular application using routine methods, as described below. It should also be noted that dsRNA oligonucleotides of the present invention having a duplex length of 19 or more nucleotides tolerate a single nucleotide mismatch at the 5' and/or 3' end of the molecule. Preferred dsRNAs of the present invention comprise duplex regions having the nucleotide lengths discussed above, and additionally, two 2'-deoxythymidine or two uridine residue overhangs at each 3'-terminus. The siRNA duplexes specifically exemplified in the examples presented herein are composed of 21 nucleotide sense and 21 nucleotide antisense strands, paired so as to have a 19 nucleotide duplex region and a two nucleotide 2'-deoxythymidine overhang at each 3'-terminus. Single-stranded RNAs containing self-complementary duplex regions of the nucleotide sizes noted above will contain extra nucleotides in the hairpin region.

dsRNAs encompassed by the present invention can consist of, consist essentially of, or comprise, the specific dsRNA ribonucleotide sequences disclosed herein. The phrase "consist essentially of," "consists essentially of," "consisting essentially of," or the like when applied to dsRNAs encompassed by the present invention refers to dsRNA sequences like those disclosed herein, but which contain additional nucleotides (ribonucleotides, deoxyribonucleotides, or analogs or derivatives thereof as discussed herein). Such additional nucleotides, however, do not materially affect the basic and novel characteristic(s) of these dsRNAs in modulating, attenuating, or inhibiting HCV gene expression, RNA function, or HCV infection, replication, and/or pathogenesis,

including the specific quantitative effects of these dsRNAs, compared to the corresponding parameters of the corresponding dsRNAs disclosed herein.

Synthesis of dsRNAs

The isolated dsRNAs and functionally equivalent variants thereof of the present invention can be obtained by a variety of techniques known in the art, including recombinantly, via enzymatic synthesis, via chemical synthesis, or by *in vivo* processing (cleavage) of longer dsRNA precursors introduced into host cells followed by extraction and purification of the resulting cleavage products from such cells. Single strands of RNA can be synthesized *in vitro*. For example, single stranded RNA can be enzymatically synthesized from the PCR products of a DNA template, preferably a cloned cDNA template. Provided the sequence of the target gene or other target polynucleotide sequence is known, a cloned cDNA template can be readily made from target cell RNA using reverse-transcriptase polymerase chain reaction (RT-PCR) to generate a cDNA fragment, followed by cloning the cDNA fragment into a suitable vector. Preferably, the vector is designed to allow the generation of complementary forward and reverse PCR products. The vector pGEM-T (Promega, Madison WI) is well suited for use in the method because it contains a cloning site positioned between oppositely oriented promoters (i.e., T7 and SP6 promoters; the T3 promoter can also be used). After purification of the PCR products, complementary single stranded RNAs are synthesized, in separate reactions, from the DNA templates via RT-PCR using two different RNA polymerases (e.g., in the case of pGEM-T, T7 polymerase and SP6 polymerase). RNAse-free DNAse is added to remove the DNA template, then the single-stranded RNA is purified.

Single strands of RNA can also be produced enzymatically using, for example, T3 and T7 RNA polymerases (Parrish et al., *Mol. Cell* 6:1077-1087 (2000)), or by partial/total organic synthesis using, for example, 2'-*O*-(triisopropyl)silyloxymethyl chemistry (Xeragon AG, Zurich, Switzerland). The use of *in vitro* enzymatic or organic synthesis allows the introduction of any desired phosphate analog, sugar, base, ribonucleotide, or combination thereof, etc. The RNA strands may or may not be polyadenylated, and the RNA strands may or may not be capable of being translated into

a polypeptide by a cell's translational apparatus. Preferably, purification of RNA is performed without the use of phenol or chloroform.

Synthesis and purification of stable RNA strands resistant to endogenous nucleases are described in U.S. patents 6,300,486; 6,294,664; 6,132,966; 6,022,962; 5,869,253; 5,837,855; 5,767,263; 5,686,599; 5,646,020; 5,631,359; and 5,610,054; and in PCT International Publications WO 01/16312 and WO 00/75306.

Double stranded RNA can be formed *in vitro* by mixing complementary single stranded RNAs, preferably in a molar ratio of at least about 3:7, more preferably in a molar ratio of about 4:6, and most preferably in essentially equal molar amounts, i.e., a molar ratio of about 5:5. Preferably, the single stranded RNAs are denatured prior to annealing, and the buffer in which the annealing reaction takes place contains a salt, such as potassium chloride or potassium acetate. An exemplary annealing buffer can comprise 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate. Annealing can be carried out for 1 minute at 90°C, followed by one hour at 37°C. The solution can be stored frozen at -20°C and freeze-thawed repeatedly. Prior to administration, the mixture containing the annealed, i.e., double stranded, RNA can be treated with an enzyme that is specific for single stranded RNA (for example, RNAse A or RNAse T) to confirm annealing and to degrade any remaining single stranded RNAs. Addition of the RNAse also serves to excise any overhanging ends on the dsRNA duplexes, if this is desired.

Commercial suppliers of dsRNAs include Dharmacon Research, Inc., Boulder, Colorado, and Xeragon AG, Zurich, Switzerland.

Design of dsRNAs: General Considerations

Tuschl and colleagues suggest a procedure for designing siRNAs for inducing RNAi in mammalian cells in:
http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html and
http://www.dharmacon.com/tech/tech003.html based on their analysis of silencing efficiency of siRNA duplexes as a function of the length of the siRNAs, the length of the 3'-overhangs, and the sequence in the overhangs using *Drosophila melanogaster* lysates (Tuschl et al., *Genes Dev.* 13:3191-3197 (1999); Zamore et al., *Cell* 101:25-33 (2000)).

Further work suggested that the most efficient silencing was obtained with siRNA duplexes composed of 21-nt sense and 21-nt antisense strands, paired so as to have a 19-nucleotide duplex region and a 2-nucleotide overhang at each 3'-terminus (Elbashir et al., *Nature* 411:494-498 (2001); PCT International Publication WO 02/44321). The 3'-overhang in the sense strand does not appear to provide any contribution to RNA target recognition as it is believed that the antisense siRNA strand guides this process. The use of two 2'-deoxythymidines in both 3'-overhangs may increase nuclease resistance, although siRNA duplexes with either UU or dTdT overhangs work equally well. 2'-deoxy substitutions of the 2-nt 3' overhanging ribonucleotides do not affect activity, help reduce the cost of RNA synthesis, and may enhance RNAse resistance of dsRNA duplexes.

The targeted region in an mRNA, and hence the sequence in the siRNA duplex, are selected using the following guidelines. Tuschl et al. recommend the open reading frame (ORF) region from the cDNA sequence for targeting, preferably at least 75 to 100 nucleotides downstream of the start codon. While these workers do not recommend the 5' and 3' untranslated regions (UTRs) and regions near the start codon for targeting, Jarvis et al. (*Ambion TechNotes* 8(5) (2001)) reported highly effective silencing of *c-myc* protein expression in a human cell line using an siRNA complementary to the 3' UTR of the *c-myc* RNA. In this case, structure within the targeted mRNA appeared to have minimal effect on the availability of the mRNA target and efficacy of the siRNA silencing approach. Thus, "rules" for siRNA duplex design and optimization are still evolving, and should be determined on a case by case basis.

Tuschl et al. recommend selecting the sequence of the siRNA as follows:

1. Start 75 bases downstream from the start codon;
2. Locate the first AA dimer;
3. Record the next 19 nucleotides following the AA dimer;
4. Calculate the percentage of guanosines and cytidines (G/C content) of the AA-N_{19} 21-base sequence. Ideally the G/C content is ~50%, but it must be less than 70% and greater than 30%. If the sequence does not meet this criterion, continue the search downstream to the next AA dimer until this condition is met;

5. The 21-base sequence is subjected to a BLAST-search (NCBI database) against EST libraries to ensure that only one gene is targeted. (The complement is automatically searched as well);
6. If the conditions in either step 4 or 5 are not met, repeat steps 2 - 5. If no suitable AA-N19 target is identified, search for a suitable CA-N19 target.

Although siRNA silencing can be achieved by selecting a single target within an mRNA, it may be desirable to design and employ two (or more) independent siRNA duplexes to control for specificity of the silencing effect. If the selected siRNA duplex(es) do not function as expected, one can conduct a search for sequencing errors in the gene, as well as possible genetic polymorphisms. It is possible that a single point mutation located in the paired region of an siRNA duplex may be sufficient to abolish target mRNA degradation. Tuschl et al. also recommend that a re-examination can be performed to confirm whether the cell line is from the expected species. Thirdly, a second and/or third target can be selected and the corresponding siRNA duplexes prepared.

Determination of dsRNA IC_{50} Values *In Vitro*

In vitro IC_{50} values for dsRNAs of the present invention can be determined by contacting *in vitro* varying concentrations of dsRNAs and appropriate cell lines, tissues, or organs that have been infected with HCV, and determining the quantitative effect(s) of these dsRNAs at such concentrations on parameters including, but not limited to, various steps, stages, or aspects of HCV infection, replication, and pathogenesis. Representative parameters that can be studied include, for example, cell entry (e.g., attachment; penetration); uncoating and release of the HCV genome; HCV-directed RNA synthesis, including replication of the HCV RNA genome; translation of the HCV polycistronic mRNA and HCV protein synthesis; post-translational modification of HCV proteins (e.g., proteolytic cleavage; glycosylation); intracellular transport of HCV proteins; virion production, release of viral particles (e.g., budding), and viral plaque formation in *in vitro* cell culture; inhibition of HCV replication; inhibition of target HCV enzyme(s); inhibition of production of HCV antigens; effect on surrogate markers; or any other HCV-associated parameter that is indicative of potential dsRNA therapeutic effectiveness that can be conveniently measured *in vitro*.

As used herein, the term "IC_{50}" refers to the concentration of one or more dsRNAs effective in inhibiting the selected parameter by 50% compared to the level of the same parameter observed in untreated or control cells. Buss et al. (*Antiviral Ther.* 6:1-7 (2001)) discuss quantifiable parameters obtainable *in vitro* that provide useful information regarding the potential clinical utility of antiviral drugs, and note that different *in vitro* assay systems can provide information on different parameters of pathogen biology and responsiveness to drug candidates. Buss et al. also note that *in vitro* IC_{50} values are clearly valuable for screening compounds during the drug development process.

A number of transfection reagents that facilitate efficient delivery of nucleic acids into cells are commercially available from suppliers such as Qiagen, Inc. (Valencia, CA), Mirus Corporation (Madison, WI), Invitrogen (Carlsbad, CA), etc. For example, reagents including, but not limited to, Lipofectamine™ 2000 Reagent, Lipofectamine Plus™ Reagent, Lipofectamine™ Reagent, DMRIE-C Reagent, Cellfectin® Reagent, Lipofectin® Reagent, and Oligofectamine™ Reagent from Invitrogen, and *Trans*IT® transfection reagent from Mirus Corporation, permit transfection of nucleic acids into a wide variety cell lines. Invitrogen provides references and protocols describing transfection of numerous cells types at its web site: http://www.invitrogen.com/transfection/cell types/. Its *Guide to Eukaryotic Transfections with Cationic Lipid Reagents* that can be found there describes, *inter alia*, maintenance and preparation of cells, troubleshooting suggestions, products and protocols for delivering nucleic acids into eukaryotic cells by transfection, recommendations for cationic lipid reagents for high efficiency transfection of numerous cell lines, and a list of references.

A typical protocol that can be used as a starting point for developing an *in vitro* assay for any cell line is given in Example 1, below. If necessary, the ordinary skilled artisan can employ the information available from Invitrogen, described above, and other published sources to optimize viral infection, nucleic acid transfection, and assay conditions for the present dsRNAs. Appropriate cell lines for *in vitro* assay of dsRNA activity against HCV are disclosed in the references cited herein. Other cell lines that may be useful in such assays can be found by reviewing the literature.

For the determination of IC_{50} values *in vitro*, the amount of dsRNA applied can be in the range of from about 0.0001 nM to about 300 μM, more preferably from about

0.0001 nM to about 200μM, more preferably from about 0.0001 nM to about 100 μM, more preferably from about 0.0001 nM to about 10 μM, more preferably from about 0.0001 nM to about 1 μM, and even more preferably from about 0.0001 nM to about 100 nM, more preferably from about 0.0001 nM to about 10 nM, and even more preferably from about 0.0001 nM to about 1 nM. In the *in vitro* cell-based assay disclosed in Example 1, this represents a ratio of the number of dsRNA molecules to the number of cells of about 30 to about 10^{12}, about 30 to about 6×10^{11}, about 30 to about 3×10^{11}, about 30 to about 3×10^{10}, about 30 to about 3×10^{9}, about 30 to about 3×10^{8}, about 30 to about 3×10^{7}, about 30 to about 3×10^{6}, and about 30 to about 3×10^{5}, respectively. Preferred dsRNAs of the present invention have an IC_{50} *in vitro* of from about 0.0001 nM to about 1 μM, more preferably from about 0.0001 nM to about 100 nM, more preferably from about 0.0001 nM to about 50 nM, more preferably from about 0.0001 nM to about 25 nM, more preferably from about 0.0001 nM to about 10 nM, more preferably from about 0.0001 nM to about 5 nM, and even more preferably from about 0.0001 nM to about 1 nM.

Given the state of the art as evidenced by the references cited herein, it should be possible for one of ordinary skill to either adapt currently existing cell-based assays, or develop completely novel *in vitro* assays, to determine IC_{50} values for the dsRNAs disclosed herein without undue experimentation.

Design of dsRNA Oligonucleotides for Treating HCV Infections

dsRNAs effective in preventing or inhibiting HCV infection, replication, and/or pathogenesis, including HCV gene expression, can have a duplex length of about 19 ribonucleotide base pairs, with a G+C content in the range of from about 30% to about 70%, preferably from about 40% to about 60%, and two 3' overhanging 2'-deoxythymidine (or uridine) residues on each strand. That a G+C content of about 40% to about 60% may be preferred is determined by analysis of preliminary results obtained with an initial series of dsRNAs tested for their capacity to inhibit HCV in the cell-based assay described in Example 1, below.

Based on these observations, one can predict from the nucleotide sequences of different HCV genomes the sequence composition of dsRNAs with potentially broad utility as HCV antiviral therapeutics as follows.

Multiple strains of HCV exist, and these strains all have similar genomic sequences. Alignment of the protein coding regions of these nucleotide sequences using bioinformatics tools such as Clustal W (v. 1.81; Thompson et al., *Nucleic Acids Res.* 22(22):4673-80 (1994)) allows one to design dsRNAs that are homologous to target polynucleotide regions of multiple HCV strains.

GenBank currently contains the complete nucleotide sequences of 147 unique HCV isolates, as follows:

D28917, AB049091, M96362, AF207766, AF207772, AJ000009, AB049096, D10988, AF139594, AF207757, AY051292, D10749, AF165059, AJ238800, AF207762, M58335, U01214, AF207755, AF011751, AB016785, AF165064, D45172, AF238483, AF207773, AF165050, AB049090, D50483, D30613, AF176573, AF238481, AF207754, D50482, AB049089, X76918, AF238486, D84262, AF165054, AB047643, D50484, AF165051, D10934, AB049093, D14484, AF165056, AB047640, D63857, D00944, AF165061, D84264, AF356827, AF054249, AB049088, AB049092, AF009606, AF177040, AB049101, M67463, D63822, AF165052, D14853, X61596, AF169005, D50485, AF165058, AF207767, AB049099, AB031663, AF207753, D50480, AF207758, AF207763, AF165045, U16362, Y13184, AF271632, AF011752, AB049095, AY045702, D11355, AF207760, AF165060, AF011753, D17763, D11168, AF046866, D10750, AF208024, D84265, AB047641, S62220, AF238482, AF207765, AB030907, Y11604, AB047645, AF165049, AF165047, D49374, AF207768, NC_001433, AF177039, AF054248, AF054247, AF238485, D89872, AF207769, Y12083, AJ132996, AB047639, AJ278830, AF165046, AF207770, AF207752, AB049094, AF207759, D89815, D50409, AF333324, AF165063, D50481, AF165062, AB047642, AB049098, AF238484, AF207774, AB047644, D13558, AF165057, AF177036, AF207764, U45476, AF165055, D85516, AJ238799, AF207761, AB049097, AJ132997, D84263, AF207771, AB049087, AF165048, M62321, AB049100, AF290978, AF165053, AF207756, D63821.

Computational alignment of the protein coding regions of these RNA sequences shows those regions containing 19 to 25 contiguous ribonucleotides conserved among multiple strains. To determine these 19-25 basepair contiguous

polyribonucleotides, a Clustal W multiple sequence alignment is made using the Clustal W v. 1.81 default parameters provided by this publicly available program.

This alignment permits determination of polyribonucleotides present in all or most of the individual HCV genomes. The HCV protein coding region alignment is performed in two steps: first the predicted HCV amino acid sequences are aligned, then the actual RNA coding sequences are aligned to the amino acid sequences codon by codon.

Because of the genetic diversity of HCV, there are no 19-25 nucleotide regions that are conserved in the coding regions in all 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%.

There are zero regions of 19 contiguous bases that are conserved in the protein coding regions in no less than 90% (133) of the 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%.

The following 13 regions of 19 contiguous bases are conserved in the coding regions in no less than 80% (118) of the 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%:

position 12-30
5'-UCCUAAACCUCAAAGAAAA-3'
3'-AGGAUUUGGAGUUUCUUUU-5'

position 13-31
5'-CCUAAACCUCAAAGAAAAA-3'
3'-GGAUUUGGAGUUUCUUUUU-5'

position 14-32
5'-CUAAACCUCAAAGAAAAAC-3'
3'-GAUUUGGAGUUUCUUUUUG-5'

position 15-33
5'-UAAACCUCAAAGAAAAACC-3'
3'-AUUUGGAGUUUCUUUUUGG-5'

position 16-34
5'-AAACCUCAAAGAAAAACCA-3'
3'-UUUGGAGUUUCUUUUUGGU-5'

position 17-35
5'-AACCUCAAAGAAAAACCAA-3'
3'-UUGGAGUUUCUUUUUGGUU-5'

position 18-36
5'-ACCUCAAAGAAAAACCAAA-3'
3'-UGGAGUUUCUUUUUGGUUU-5'

position 356-374
5'-UGGGUAAGGUCAUCGAUAC-3'
3'-ACCCAUUCCAGUAGCUAUG-5'

position 357-375
5'-GGGUAAGGUCAUCGAUACC-3'
3'-CCCAUUCCAGUAGCUAUGG-5'

position 358-376
5'-GGUAAGGUCAUCGAUACCC-3'
3'-CCAUUCCAGUAGCUAUGGG-5'

position 359-377
5'-GUAAGGUCAUCGAUACCCU-3'
3'-CAUUCCAGUAGCUAUGGGA-5'

position 391-409
5'-GCCGACCUCAUGGGGUACA-3'
3'-CGGCUGGAGUACCCCAUGU-5'

position 392-410
5'-CCGACCUCAUGGGGUACAU-3'
3'-GGCUGGAGUACCCCAUGUA-5'

The following 32 regions of 19 contiguous bases are conserved in the coding regions in no less than 70% (103) of the 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%:

position 10-28
5'-AAUCCUAAACCUCAAAGAA-3'
3'-UUAGGAUUUGGAGUUUCUU-5'

position 11-29
5'-AUCCUAAACCUCAAAGAAA-3'
3'-UAGGAUUUGGAGUUUCUUU-5'

position 12-30
5'-UCCUAAACCUCAAAGAAAA-3'
3'-AGGAUUUGGAGUUUCUUUU-5'

position 13-31
5'-CCUAAACCUCAAAGAAAAA-3'
3'-GGAUUUGGAGUUUCUUUUU-5'

position 14-32
5'-CUAAACCUCAAAGAAAAAC-3'
3'-GAUUUGGAGUUUCUUUUUG-5'

position 15-33
5'-UAAACCUCAAAGAAAAACC-3'
3'-AUUUGGAGUUUCUUUUUGG-5'

position 16-34
5'-AAACCUCAAAGAAAAACCA-3'
3'-UUUGGAGUUUCUUUUUGGU-5'

position 17-35
5'-AACCUCAAAGAAAAACCAA-3'
3'-UUGGAGUUUCUUUUUGGUU-5'

position 18-36
5'-ACCUCAAAGAAAAACCAAA-3'
3'-UGGAGUUUCUUUUUGGUUU-5'

position 22-40
5'-CAAAGAAAAACCAAACGUA-3'
3'-GUUUCUUUUUGGUUUGCAU-5'

position 24-42
5'-AAGAAAAACCAAACGUAAC-3'
3'-UUCUUUUUGGUUUGCAUUG-5'

position 25-43
5'-AGAAAAACCAAACGUAACA-3'
3'-UCUUUUUGGUUUGCAUUGU-5'

position 26-44
5'-GAAAAACCAAACGUAACAC-3'
3'-CUUUUUGGUUUGCAUUGUG-5'

position 27-45
5'-AAAAACCAAACGUAACACC-3'
3'-UUUUUGGUUUGCAUUGUGG-5'

position 30-48
5'-AACCAAACGUAACACCAAC-3'
3'-UUGGUUUGCAUUGUGGUUG-5'

position 31-49
5'-ACCAAACGUAACACCAACC-3'

3'-UGGUUUGCAUUGUGGUUGG-5'

position 32-50
5'-CCAAACGUAACACCAACCG-3'
3'-GGUUUGCAUUGUGGUUGGC-5'

position 85-103
5'-CAGAUCGUUGGUGGAGUUU-3'
3'-GUCUAGCAACCACCUCAAA-5'

position 86-104
5'-AGAUCGUUGGUGGAGUUUA-3'
3'-UCUAGCAACCACCUCAAAU-5'

position 87-105
5'-GAUCGUUGGUGGAGUUUAC-3'
3'-CUAGCAACCACCUCAAAUG-5'

position 148-166
5'-AGGAAGACUUCCGAGCGGU-3'
3'-UCCUUCUGAAGGCUCGCCA-5'

position 149-167
5'-GGAAGACUUCCGAGCGGUC-3'
3'-CCUUCUGAAGGCUCGCCAG-5'

position 277-295
5'-UGGGCAGGAUGGCUCCUGU-3'
3'-ACCCGUCCUACCGAGGACA-5'

position 278-296
5'-GGGCAGGAUGGCUCCUGUC-3'
3'-CCCGUCCUACCGAGGACAG-5'

position 356-374
5'-UGGGUAAGGUCAUCGAUAC-3'
3'-ACCCAUUCCAGUAGCUAUG-5'

position 357-375
5'-GGGUAAGGUCAUCGAUACC-3'
3'-CCCAUUCCAGUAGCUAUGG-5'

position 358-376
5'-GGUAAGGUCAUCGAUACCC-3'
3'-CCAUUCCAGUAGCUAUGGG-5'

position 359-377
5'-GUAAGGUCAUCGAUACCCU-3'

3'-CAUUCCAGUAGCUAUGGGA-5'

position 389-407
5'-UCGCCGACCUCAUGGGGUA-3'
3'-AGCGGCUGGAGUACCCCAU-5'
position 390-408
5'-CGCCGACCUCAUGGGGUAC-3'
3'-GCGGCUGGAGUACCCCAUG-5'

position 391-409
5'-GCCGACCUCAUGGGGUACA-3'
3'-CGGCUGGAGUACCCCAUGU-5'

position 392-410
5'-CCGACCUCAUGGGGUACAU-3'
3'-GGCUGGAGUACCCCAUGUA-5'

The following 154 regions of 19 contiguous bases are conserved in the coding regions in no less than 60% (89) of the 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%:

position 10-28
5'-AAUCCUAAACCUCAAAGAA-3'
3'-UUAGGAUUUGGAGUUUCUU-5'

position 11-29
5'-AUCCUAAACCUCAAAGAAA-3'
3'-UAGGAUUUGGAGUUUCUUU-5'

position 12-30
5'-UCCUAAACCUCAAAGAAAA-3'
3'-AGGAUUUGGAGUUUCUUUU-5'

position 13-31
5'-CCUAAACCUCAAAGAAAAA-3'
3'-GGAUUUGGAGUUUCUUUUU-5'

position 14-32
5'-CUAAACCUCAAAGAAAAAC-3'
3'-GAUUUGGAGUUUCUUUUUG-5'

position 15-33
5'-UAAACCUCAAAGAAAAACC-3'
3'-AUUUGGAGUUUCUUUUUGG-5'

position 16-34
5'-AAACCUCAAAGAAAAACCA-3'
3'-UUUGGAGUUUCUUUUUGGU-5'

position 17-35
5'-AACCUCAAAGAAAAACCAA-3'
3'-UUGGAGUUUCUUUUUGGUU-5'

position 18-36
5'-ACCUCAAAGAAAAACCAAA-3'
3'-UGGAGUUUCUUUUUGGUUU-5'

position 19-37
5'-CCUCAAAGAAAAACCAAAC-3'
3'-GGAGUUUCUUUUUGGUUUG-5'

position 20-38
5'-CUCAAAGAAAAACCAAACG-3'
3'-GAGUUUCUUUUUGGUUUGC-5'

position 21-39
5'-UCAAAGAAAAACCAAACGU-3'
3'-AGUUUCUUUUUGGUUUGCA-5'

position 22-40
5'-CAAAGAAAAACCAAACGUA-3'
3'-GUUUCUUUUUGGUUUGCAU-5'

position 24-42
5'-AAGAAAAACCAAACGUAAC-3'
3'-UUCUUUUUGGUUUGCAUUG-5'

position 25-43
5'-AGAAAAACCAAACGUAACA-3'
3'-UCUUUUUGGUUUGCAUUGU-5'

position 26-44
5'-GAAAAACCAAACGUAACAC-3'
3'-CUUUUUGGUUUGCAUUGUG-5'

position 27-45
5'-AAAAACCAAACGUAACACC-3'
3'-UUUUUGGUUUGCAUUGUGG-5'

position 28-46
5'-AAAACCAAACGUAACACCA-3'
3'-UUUUGGUUUGCAUUGUGGU-5'

position 29-47
5'-AAACCAAACGUAACACCAA-3'
3'-UUUGGUUUGCAUUGUGGUU-5'

position 30-48
5'-AACCAAACGUAACACCAAC-3'
3'-UUGGUUUGCAUUGUGGUUG-5'

position 31-49
5'-ACCAAACGUAACACCAACC-3'
3'-UGGUUUGCAUUGUGGUUGG-5'

position 32-50
5'-CCAAACGUAACACCAACCG-3'
3'-GGUUUGCAUUGUGGUUGGC-5'

position 33-51
5'-CAAACGUAACACCAACCGC-3'
3'-GUUUGCAUUGUGGUUGGCG-5'

position 34-52
5'-AAACGUAACACCAACCGCC-3'
3'-UUUGCAUUGUGGUUGGCGG-5'

position 35-53
5'-AACGUAACACCAACCGCCG-3'
3'-UUGCAUUGUGGUUGGCGGC-5'

position 39-57
5'-UAACACCAACCGCCGCCCA-3'
3'-AUUGUGGUUGGCGGCGGGU-5'

position 40-58
5'-AACACCAACCGCCGCCCAC-3'
3'-UUGUGGUUGGCGGCGGGUG-5'

position 41-59
5'-ACACCAACCGCCGCCCACA-3'
3'-UGUGGUUGGCGGCGGGUGU-5'

position 52-70
5'-CGCCCACAGGACGUCAAGU-3'
3'-GCGGGUGUCCUGCAGUUCA-5'

position 53-71
5'-GCCCACAGGACGUCAAGUU-3'
3'-CGGGUGUCCUGCAGUUCAA-5'

position 54-72
5'-CCCACAGGACGUCAAGUUC-3'
3'-GGGUGUCCUGCAGUUCAAG-5'

position 55-73
5'-CCACAGGACGUCAAGUUCC-3'
3'-GGUGUCCUGCAGUUCAAGG-5'

position 56-74
5'-CACAGGACGUCAAGUUCCC-3'
3'-GUGUCCUGCAGUUCAAGGG-5'

position 57-75
5'-ACAGGACGUCAAGUUCCCG-3'
3'-UGUCCUGCAGUUCAAGGGC-5'

position 58-76
5'-CAGGACGUCAAGUUCCCGG-3'
3'-GUCCUGCAGUUCAAGGGCC-5'

position 59-77
5'-AGGACGUCAAGUUCCCGGG-3'
3'-UCCUGCAGUUCAAGGGCCC-5'

position 67-85
5'-AAGUUCCCGGGCGGUGGUC-3'
3'-UUCAAGGGCCCGCCACCAG-5'

position 68-86
5'-AGUUCCCGGGCGGUGGUCA-3'
3'-UCAAGGGCCCGCCACCAGU-5'

position 70-88
5'-UUCCCGGGCGGUGGUCAGA-3'
3'-AAGGGCCCGCCACCAGUCU-5'

position 71-89
5'-UCCCGGGCGGUGGUCAGAU-3'
3'-AGGGCCCGCCACCAGUCUA-5'

position 74-92
5'-CGGGCGGUGGUCAGAUCGU-3'
3'-GCCCGCCACCAGUCUAGCA-5'

position 82-100
5'-GGUCAGAUCGUUGGUGGAG-3'
3'-CCAGUCUAGCAACCACCUC-5'

position 83-101
5'-GUCAGAUCGUUGGUGGAGU-3'
3'-CAGUCUAGCAACCACCUCA-5'

position 84-102
5'-UCAGAUCGUUGGUGGAGUU-3'
3'-AGUCUAGCAACCACCUCAA-5'

position 85-103
5'-CAGAUCGUUGGUGGAGUUU-3'
3'-GUCUAGCAACCACCUCAAA-5'

position 86-104
5'-AGAUCGUUGGUGGAGUUUA-3'
3'-UCUAGCAACCACCUCAAAU-5'

position 87-105
5'-GAUCGUUGGUGGAGUUUAC-3'
3'-CUAGCAACCACCUCAAAUG-5'

position 88-106
5'-AUCGUUGGUGGAGUUUACC-3'
3'-UAGCAACCACCUCAAAUGG-5'

position 89-107
5'-UCGUUGGUGGAGUUUACCU-3'
3'-AGCAACCACCUCAAAUGGA-5'

position 90-108
5'-CGUUGGUGGAGUUUACCUG-3'
3'-GCAACCACCUCAAAUGGAC-5'

position 91-109
5'-GUUGGUGGAGUUUACCUGU-3'
3'-CAACCACCUCAAAUGGACA-5'

position 92-110
5'-UUGGUGGAGUUUACCUGUU-3'
3'-AACCACCUCAAAUGGACAA-5'

position 93-111
5'-UGGUGGAGUUUACCUGUUG-3'
3'-ACCACCUCAAAUGGACAAC-5'

position 94-112
5'-GGUGGAGUUUACCUGUUGC-3'
3'-CCACCUCAAAUGGACAACG-5'

position 95-113
5'-GUGGAGUUUACCUGUUGCC-3'
3'-CACCUCAAAUGGACAACGG-5'

5'-UGGAGUUUACCUGUUGCCG-3'
3'-ACCUCAAAUGGACAACGGC-5'

position 97-115
5'-GGAGUUUACCUGUUGCCGC-3'
3'-CCUCAAAUGGACAACGGCG-5'

position 98-116
5'-GAGUUUACCUGUUGCCGCG-3'
3'-CUCAAAUGGACAACGGCGC-5'

position 99-117
5'-AGUUUACCUGUUGCCGCGC-3'
3'-UCAAAUGGACAACGGCGCG-5'

position 100-118
5'-GUUUACCUGUUGCCGCGCA-3'
3'-CAAAUGGACAACGGCGCGU-5'

position 101-119
5'-UUUACCUGUUGCCGCGCAG-3'
3'-AAAUGGACAACGGCGCGUC-5'

position 102-120
5'-UUACCUGUUGCCGCGCAGG-3'
3'-AAUGGACAACGGCGCGUCC-5'

position 103-121
5'-UACCUGUUGCCGCGCAGGG-3'
3'-AUGGACAACGGCGCGUCCC-5'

position 127-145
5'-AGGUUGGGUGUGCGCGCGA-3'
3'-UCCAACCCACACGCGCGCU-5'

position 129-147
5'-GUUGGGUGUGCGCGCGACU-3'
3'-CAACCCACACGCGCGCUGA-5'

position 130-148
5'-UUGGGUGUGCGCGCGACUA-3'
3'-AACCCACACGCGCGCUGAU-5'

position 131-149

5'-UGGGUGUGCGCGCGACUAG-3'
3'-ACCCACACGCGCGCUGAUC-5'

position 133-151
5'-GGUGUGCGCGCGACUAGGA-3'
3'-CCACACGCGCGCUGAUCCU-5'

position 134-152
5'-GUGUGCGCGCGACUAGGAA-3'
3'-CACACGCGCGCUGAUCCUU-5'

position 135-153
5'-UGUGCGCGCGACUAGGAAG-3'
3'-ACACGCGCGCUGAUCCUUC-5'

position 136-154
5'-GUGCGCGCGACUAGGAAGA-3'
3'-CACGCGCGCUGAUCCUUCU-5'

position 137-155
5'-UGCGCGCGACUAGGAAGAC-3'
3'-ACGCGCGCUGAUCCUUCUG-5'

position 138-156
5'-GCGCGCGACUAGGAAGACU-3'
3'-CGCGCGCUGAUCCUUCUGA-5'

position 139-157
5'-CGCGCGACUAGGAAGACUU-3'
3'-GCGCGCUGAUCCUUCUGAA-5'

position 140-158
5'-GCGCGACUAGGAAGACUUC-3'
3'-CGCGCUGAUCCUUCUGAAG-5'

position 141-159
5'-CGCGACUAGGAAGACUUCC-3'
3'-GCGCUGAUCCUUCUGAAGG-5'

position 142-160
5'-GCGACUAGGAAGACUUCCG-3'
3'-CGCUGAUCCUUCUGAAGGC-5'

position 143-161
5'-CGACUAGGAAGACUUCCGA-3'
3'-GCUGAUCCUUCUGAAGGCU-5'

position 146-164
5'-CUAGGAAGACUUCCGAGCG-3'
3'-GAUCCUUCUGAAGGCUCGC-5'

position 147-165
5'-UAGGAAGACUUCCGAGCGG-3'
3'-AUCCUUCUGAAGGCUCGCC-5'

position 148-166
5'-AGGAAGACUUCCGAGCGGU-3'
3'-UCCUUCUGAAGGCUCGCCA-5'

position 149-167
5'-GGAAGACUUCCGAGCGGUC-3'
3'-CCUUCUGAAGGCUCGCCAG-5'

position 150-168
5'-GAAGACUUCCGAGCGGUCG-3'
3'-CUUCUGAAGGCUCGCCAGC-5'

position 151-169
5'-AAGACUUCCGAGCGGUCGC-3'
3'-UUCUGAAGGCUCGCCAGCG-5'

position 152-170
5'-AGACUUCCGAGCGGUCGCA-3'
3'-UCUGAAGGCUCGCCAGCGU-5'

position 153-171
5'-GACUUCCGAGCGGUCGCAA-3'
3'-CUGAAGGCUCGCCAGCGUU-5'

position 154-172
5'-ACUUCCGAGCGGUCGCAAC-3'
3'-UGAAGGCUCGCCAGCGUUG-5'

position 155-173
5'-CUUCCGAGCGGUCGCAACC-3'
3'-GAAGGCUCGCCAGCGUUGG-5'

position 156-174
5'-UUCCGAGCGGUCGCAACCU-3'
3'-AAGGCUCGCCAGCGUUGGA-5'

position 157-175
5'-UCCGAGCGGUCGCAACCUC-3'
3'-AGGCUCGCCAGCGUUGGAG-5'

position 169-187
5'-CAACCUCGUGGAAGGCGAC-3'
3'-GUUGGAGCACCUUCCGCUG-5'

position 170-188
5'-AACCUCGUGGAAGGCGACA-3'
3'-UUGGAGCACCUUCCGCUGU-5'

position 238-256
5'-GGGUACCCUUGGCCCCUCU-3'
3'-CCCAUGGGAACCGGGGAGA-5'

position 239-257
5'-GGUACCCUUGGCCCCUCUA-3'
3'-CCAUGGGAACCGGGGAGAU-5'

position 272-290
5'-UGGGGUGGGCAGGAUGGCU-3'
3'-ACCCCACCCGUCCUACCGA-5'

position 275-293
5'-GGUGGGCAGGAUGGCUCCU-3'
3'-CCACCCGUCCUACCGAGGA-5'

position 276-294
5'-GUGGGCAGGAUGGCUCCUG-3'
3'-CACCCGUCCUACCGAGGAC-5'

position 277-295
5'-UGGGCAGGAUGGCUCCUGU-3'
3'-ACCCGUCCUACCGAGGACA-5'

position 278-296
5'-GGGCAGGAUGGCUCCUGUC-3'
3'-CCCGUCCUACCGAGGACAG-5'

position 352-370
5'-AAUUUGGGUAAGGUCAUCG-3'
3'-UUAAACCCAUUCCAGUAGC-5'

position 353-371
5'-AUUUGGGUAAGGUCAUCGA-3'
3'-UAAACCCAUUCCAGUAGCU-5'

position 355-373
5'-UUGGGUAAGGUCAUCGAUA-3'
3'-AACCCAUUCCAGUAGCUAU-5'

position 356-374
5'-UGGGUAAGGUCAUCGAUAC-3'
3'-ACCCAUUCCAGUAGCUAUG-5'

position 357-375
5'-GGGUAAGGUCAUCGAUACC-3'
3'-CCCAUUCCAGUAGCUAUGG-5'

position 358-376
5'-GGUAAGGUCAUCGAUACCC-3'
3'-CCAUUCCAGUAGCUAUGGG-5'

position 359-377
5'-GUAAGGUCAUCGAUACCCU-3'
3'-CAUUCCAGUAGCUAUGGGA-5'

position 382-400
5'-UGCGGCUUCGCCGACCUCA-3'
3'-ACGCCGAAGCGGCUGGAGU-5'

position 383-401
5'-GCGGCUUCGCCGACCUCAU-3'
3'-CGCCGAAGCGGCUGGAGUA-5'

position 384-402
5'-CGGCUUCGCCGACCUCAUG-3'
3'-GCCGAAGCGGCUGGAGUAC-5'

position 385-403
5'-GGCUUCGCCGACCUCAUGG-3'
3'-CCGAAGCGGCUGGAGUACC-5'

position 386-404
5'-GCUUCGCCGACCUCAUGGG-3'
3'-CGAAGCGGCUGGAGUACCC-5'

position 387-405
5'-CUUCGCCGACCUCAUGGGG-3'
3'-GAAGCGGCUGGAGUACCCC-5'

position 388-406
5'-UUCGCCGACCUCAUGGGGU-3'
3'-AAGCGGCUGGAGUACCCCA-5'

position 389-407
5'-UCGCCGACCUCAUGGGGUA-3'
3'-AGCGGCUGGAGUACCCCAU-5'

position 390-408
5'-CGCCGACCUCAUGGGGUAC-3'
3'-GCGGCUGGAGUACCCCAUG-5'

position 391-409
5'-GCCGACCUCAUGGGGUACA-3'
3'-CGGCUGGAGUACCCCAUGU-5'

position 392-410
5'-CCGACCUCAUGGGGUACAU-3'
3'-GGCUGGAGUACCCCAUGUA-5'

position 511-529
5'-GGUUGCUCUUUCUCUAUCU-3'
3'-CCAACGAGAAAGAGAUAGA-5'

position 512-530
5'-GUUGCUCUUUCUCUAUCUU-3'
3'-CAACGAGAAAGAGAUAGAA-5'

position 513-531
5'-UUGCUCUUUCUCUAUCUUC-3'
3'-AACGAGAAAGAGAUAGAAG-5'

position 514-532
5'-UGCUCUUUCUCUAUCUUCC-3'
3'-ACGAGAAAGAGAUAGAAGG-5'

position 515-533
5'-GCUCUUUCUCUAUCUUCCU-3'
3'-CGAGAAAGAGAUAGAAGGA-5'

position 961-979
5'-UGGGAUAUGAUGAUGAACU-3'
3'-ACCCUAUACUACUACUUGA-5'

position 962-980
5'-GGGAUAUGAUGAUGAACUG-3'
3'-CCCUAUACUACUACUUGAC-5'

position 963-981
5'-GGAUAUGAUGAUGAACUGG-3'
3'-CCUAUACUACUACUUGACC-5'

position 964-982
5'-GAUAUGAUGAUGAACUGGU-3'
3'-CUAUACUACUACUUGACCA-5'

position 965-983
5'-AUAUGAUGAUGAACUGGUC-3'
3'-UAUACUACUACUUGACCAG-5'

position 4045-4063
5'-CAAGCGGAGACGGCUGGAG-3'
3'-GUUCGCCUCUGCCGACCUC-5'

position 4046-4064
5'-AAGCGGAGACGGCUGGAGC-3'
3'-UUCGCCUCUGCCGACCUCG-5'

position 5779-5797
5'-GUGCAGUGGAUGAACCGGC-3'
3'-CACGUCACCUACUUGGCCG-5'

position 5780-5798
5'-UGCAGUGGAUGAACCGGCU-3'
3'-ACGUCACCUACUUGGCCGA-5'

position 5782-5800
5'-CAGUGGAUGAACCGGCUGA-3'
3'-GUCACCUACUUGGCCGACU-5'

position 5783-5801
5'-AGUGGAUGAACCGGCUGAU-3'
3'-UCACCUACUUGGCCGACUA-5'

position 5784-5802
5'-GUGGAUGAACCGGCUGAUA-3'
3'-CACCUACUUGGCCGACUAU-5'

position 5785-5803
5'-UGGAUGAACCGGCUGAUAG-3'
3'-ACCUACUUGGCCGACUAUC-5'

position 5786-5804
5'-GGAUGAACCGGCUGAUAGC-3'
3'-CCUACUUGGCCGACUAUCG-5'

position 5787-5805
5'-GAUGAACCGGCUGAUAGCG-3'
3'-CUACUUGGCCGACUAUCGC-5'

position 5788-5806
5'-AUGAACCGGCUGAUAGCGU-3'
3'-UACUUGGCCGACUAUCGCA-5'

position 5789-5807
5'-UGAACCGGCUGAUAGCGUU-3'
3'-ACUUGGCCGACUAUCGCAA-5'

position 7615-7633
5'-AAGGAGAUGAAGGCGAAGG-3'
3'-UUCCUCUACUUCCGCUUCC-5'

position 7616-7634
5'-AGGAGAUGAAGGCGAAGGC-3'
3'-UCCUCUACUUCCGCUUCCG-5'

position 7617-7635
5'-GGAGAUGAAGGCGAAGGCG-3'
3'-CCUCUACUUCCGCUUCCGC-5'

position 7618-7636
5'-GAGAUGAAGGCGAAGGCGU-3'
3'-CUCUACUUCCGCUUCCGCA-5'

position 7619-7637
5'-AGAUGAAGGCGAAGGCGUC-3'
3'-UCUACUUCCGCUUCCGCAG-5'

position 8424-8442
5'-CUUCACGGAGGCUAUGACU-3'
3'-GAAGUGCCUCCGAUACUGA-5'

position 8425-8443
5'-UUCACGGAGGCUAUGACUA-3'
3'-AAGUGCCUCCGAUACUGAU-5'

position 8426-8444
5'-UCACGGAGGCUAUGACUAG-3'
3'-AGUGCCUCCGAUACUGAUC-5'

position 8427-8445
5'-CACGGAGGCUAUGACUAGG-3'
3'-GUGCCUCCGAUACUGAUCC-5'

position 8428-8446
5'-ACGGAGGCUAUGACUAGGU-3'
3'-UGCCUCCGAUACUGAUCCA-5'

position 8429-8447

5'-CGGAGGCUAUGACUAGGUA-3'
3'-GCCUCCGAUACUGAUCCAU-5'

position 9078-9096
5'-CGGGGGAGACAUAUAUCAC-3'
3'-GCCCCCUCUGUAUAUAGUG-5'

position 9079-9097
5'-GGGGGAGACAUAUAUCACA-3'
3'-CCCCCUCUGUAUAUAGUGU-5'

position 9080-9098
5'-GGGGAGACAUAUAUCACAG-3'
3'-CCCCUCUGUAUAUAGUGUC-5'

position 9081-9099
5'-GGGAGACAUAUAUCACAGC-3'
3'-CCCUCUGUAUAUAGUGUCG-5'

The following 424 regions of 19 contiguous bases are conserved in the coding regions in no less than 50% (74) of the 147 complete genomes that comprise the aforementioned set of HCV RNA sequences with G+C% between 30% and 70%:

position 10-28
5'-AAUCCUAAACCUCAAAGAA-3'
3'-UUAGGAUUUGGAGUUUCUU-5'

position 11-29
5'-AUCCUAAACCUCAAAGAAA-3'
3'-UAGGAUUUGGAGUUUCUUU-5'

position 12-30
5'-UCCUAAACCUCAAAGAAAA-3'
3'-AGGAUUUGGAGUUUCUUUU-5'

position 13-31
5'-CCUAAACCUCAAAGAAAAA-3'
3'-GGAUUUGGAGUUUCUUUUU-5'

position 14-32
5'-CUAAACCUCAAAGAAAAAC-3'
3'-GAUUUGGAGUUUCUUUUUG-5'

position 15-33
5'-UAAACCUCAAAGAAAAACC-3'
3'-AUUUGGAGUUUCUUUUUGG-5'

position 16-34
5'-AAACCUCAAAGAAAAACCA-3'
3'-UUUGGAGUUUCUUUUUGGU-5'

position 17-35
5'-AACCUCAAAGAAAAACCAA-3'
3'-UUGGAGUUUCUUUUUGGUU-5'

position 18-36
5'-ACCUCAAAGAAAAACCAAA-3'
3'-UGGAGUUUCUUUUUGGUUU-5'

position 19-37
5'-CCUCAAAGAAAAACCAAAC-3'
3'-GGAGUUUCUUUUUGGUUUG-5'

position 20-38
5'-CUCAAAGAAAAACCAAACG-3'
3'-GAGUUUCUUUUUGGUUUGC-5'

position 21-39
5'-UCAAAGAAAAACCAAACGU-3'
3'-AGUUUCUUUUUGGUUUGCA-5'

position 22-40
5'-CAAAGAAAAACCAAACGUA-3'
3'-GUUUCUUUUUGGUUUGCAU-5'

position 24-42
5'-AAGAAAAACCAAACGUAAC-3'
3'-UUCUUUUUGGUUUGCAUUG-5'

position 25-43
5'-AGAAAAACCAAACGUAACA-3'
3'-UCUUUUUGGUUUGCAUUGU-5'

position 26-44
5'-GAAAAACCAAACGUAACAC-3'
3'-CUUUUUGGUUUGCAUUGUG-5'

position 27-45
5'-AAAAACCAAACGUAACACC-3'
3'-UUUUUGGUUUGCAUUGUGG-5'

position 28-46
5'-AAAACCAAACGUAACACCA-3'
3'-UUUUGGUUUGCAUUGUGGU-5'

position 29-47
5'-AAACCAAACGUAACACCAA-3'
3'-UUUGGUUUGCAUUGUGGUU-5'

position 30-48
5'-AACCAAACGUAACACCAAC-3'
3'-UUGGUUUGCAUUGUGGUUG-5'

position 31-49
5'-ACCAAACGUAACACCAACC-3'
3'-UGGUUUGCAUUGUGGUUGG-5'

position 32-50
5'-CCAAACGUAACACCAACCG-3'
3'-GGUUUGCAUUGUGGUUGGC-5'

position 33-51
5'-CAAACGUAACACCAACCGC-3'
3'-GUUUGCAUUGUGGUUGGCG-5'

position 34-52
5'-AAACGUAACACCAACCGCC-3'
3'-UUUGCAUUGUGGUUGGCGG-5'

position 35-53
5'-AACGUAACACCAACCGCCG-3'
3'-UUGCAUUGUGGUUGGCGGC-5'

position 36-54
5'-ACGUAACACCAACCGCCGC-3'
3'-UGCAUUGUGGUUGGCGGCG-5'

position 37-55
5'-CGUAACACCAACCGCCGCC-3'
3'-GCAUUGUGGUUGGCGGCGG-5'

position 38-56
5'-GUAACACCAACCGCCGCCC-3'
3'-CAUUGUGGUUGGCGGCGGG-5'

position 39-57
5'-UAACACCAACCGCCGCCCA-3'
3'-AUUGUGGUUGGCGGCGGGU-5'

position 40-58
5'-AACACCAACCGCCGCCCAC-3'
3'-UUGUGGUUGGCGGCGGGUG-5'

position 41-59
5'-ACACCAACCGCCGCCCACA-3'
3'-UGUGGUUGGCGGCGGGUGU-5'

position 50-68
5'-GCCGCCCACAGGACGUCAA-3'
3'-CGGCGGGUGUCCUGCAGUU-5'

position 51-69
5'-CCGCCCACAGGACGUCAAG-3'
3'-GGCGGGUGUCCUGCAGUUC-5'

position 52-70
5'-CGCCCACAGGACGUCAAGU-3'
3'-GCGGGUGUCCUGCAGUUCA-5'

position 53-71
5'-GCCCACAGGACGUCAAGUU-3'
3'-CGGGUGUCCUGCAGUUCAA-5'

position 54-72
5'-CCCACAGGACGUCAAGUUC-3'
3'-GGGUGUCCUGCAGUUCAAG-5'

position 55-73
5'-CCACAGGACGUCAAGUUCC-3'
3'-GGUGUCCUGCAGUUCAAGG-5'

position 56-74
5'-CACAGGACGUCAAGUUCCC-3'
3'-GUGUCCUGCAGUUCAAGGG-5'

position 57-75
5'-ACAGGACGUCAAGUUCCCG-3'
3'-UGUCCUGCAGUUCAAGGGC-5'

position 58-76
5'-CAGGACGUCAAGUUCCCGG-3'
3'-GUCCUGCAGUUCAAGGGCC-5'

position 59-77
5'-AGGACGUCAAGUUCCCGGG-3'
3'-UCCUGCAGUUCAAGGGCCC-5'

position 60-78
5'-GGACGUCAAGUUCCCGGGC-3'
3'-CCUGCAGUUCAAGGGCCCG-5'

position 61-79
5'-GACGUCAAGUUCCCGGGCG-3'
3'-CUGCAGUUCAAGGGCCCGC-5'

position 62-80
5'-ACGUCAAGUUCCCGGGCGG-3'
3'-UGCAGUUCAAGGGCCCGCC-5'

position 63-81
5'-CGUCAAGUUCCCGGGCGGU-3'
3'-GCAGUUCAAGGGCCCGCCA-5'

position 64-82
5'-GUCAAGUUCCCGGGCGGUG-3'
3'-CAGUUCAAGGGCCCGCCAC-5'

position 65-83
5'-UCAAGUUCCCGGGCGGUGG-3'
3'-AGUUCAAGGGCCCGCCACC-5'

position 66-84
5'-CAAGUUCCCGGGCGGUGGU-3'
3'-GUUCAAGGGCCCGCCACCA-5'

position 67-85
5'-AAGUUCCCGGGCGGUGGUC-3'
3'-UUCAAGGGCCCGCCACCAG-5'

position 68-86
5'-AGUUCCCGGGCGGUGGUCA-3'
3'-UCAAGGGCCCGCCACCAGU-5'

position 70-88
5'-UUCCCGGGCGGUGGUCAGA-3'
3'-AAGGGCCCGCCACCAGUCU-5'

position 71-89
5'-UCCCGGGCGGUGGUCAGAU-3'
3'-AGGGCCCGCCACCAGUCUA-5'

position 74-92
5'-CGGGCGGUGGUCAGAUCGU-3'
3'-GCCCGCCACCAGUCUAGCA-5'

position 75-93
5'-GGGCGGUGGUCAGAUCGUU-3'
3'-CCCGCCACCAGUCUAGCAA-5'

position 76-94
5'-GGCGGUGGUCAGAUCGUUG-3'
3'-CCGCCACCAGUCUAGCAAC-5'

position 77-95
5'-GCGGUGGUCAGAUCGUUGG-3'
3'-CGCCACCAGUCUAGCAACC-5'

position 78-96
5'-CGGUGGUCAGAUCGUUGGU-3'
3'-GCCACCAGUCUAGCAACCA-5'

position 79-97
5'-GGUGGUCAGAUCGUUGGUG-3'
3'-CCACCAGUCUAGCAACCAC-5'

position 80-98
5'-GUGGUCAGAUCGUUGGUGG-3'
3'-CACCAGUCUAGCAACCACC-5'

position 81-99
5'-UGGUCAGAUCGUUGGUGGA-3'
3'-ACCAGUCUAGCAACCACCU-5'

position 82-100
5'-GGUCAGAUCGUUGGUGGAG-3'
3'-CCAGUCUAGCAACCACCUC-5'

position 83-101
5'-GUCAGAUCGUUGGUGGAGU-3'
3'-CAGUCUAGCAACCACCUCA-5'

position 84-102
5'-UCAGAUCGUUGGUGGAGUU-3'
3'-AGUCUAGCAACCACCUCAA-5'

position 85-103
5'-CAGAUCGUUGGUGGAGUUU-3'
3'-GUCUAGCAACCACCUCAAA-5'

position 86-104
5'-AGAUCGUUGGUGGAGUUUA-3'
3'-UCUAGCAACCACCUCAAAU-5'

position 87-105
5'-GAUCGUUGGUGGAGUUUAC-3'
3'-CUAGCAACCACCUCAAAUG-5'
position 88-106
5'-AUCGUUGGUGGAGUUUACC-3'
3'-UAGCAACCACCUCAAAUGG-5'

position 89-107
5'-UCGUUGGUGGAGUUUACCU-3'
3'-AGCAACCACCUCAAAUGGA-5'

position 90-108
5'-CGUUGGUGGAGUUUACCUG-3'
3'-GCAACCACCUCAAAUGGAC-5'

position 91-109
5'-GUUGGUGGAGUUUACCUGU-3'
3'-CAACCACCUCAAAUGGACA-5'

position 92-110
5'-UUGGUGGAGUUUACCUGUU-3'
3'-AACCACCUCAAAUGGACAA-5'

position 93-111
5'-UGGUGGAGUUUACCUGUUG-3'
3'-ACCACCUCAAAUGGACAAC-5'

position 94-112
5'-GGUGGAGUUUACCUGUUGC-3'
3'-CCACCUCAAAUGGACAACG-5'

position 95-113
5'-GUGGAGUUUACCUGUUGCC-3'
3'-CACCUCAAAUGGACAACGG-5'

position 96-114
5'-UGGAGUUUACCUGUUGCCG-3'
3'-ACCUCAAAUGGACAACGGC-5'

position 97-115
5'-GGAGUUUACCUGUUGCCGC-3'
3'-CCUCAAAUGGACAACGGCG-5'

position 98-116

5'-GAGUUUACCUGUUGCCGCG-3'
3'-CUCAAAUGGACAACGGCGC-5'

position 99-117
5'-AGUUUACCUGUUGCCGCGC-3'
3'-UCAAAUGGACAACGGCGCG-5'
position 100-118
5'-GUUUACCUGUUGCCGCGCA-3'
3'-CAAAUGGACAACGGCGCGU-5'

position 101-119
5'-UUUACCUGUUGCCGCGCAG-3'
3'-AAAUGGACAACGGCGCGUC-5'

position 102-120
5'-UUACCUGUUGCCGCGCAGG-3'
3'-AAUGGACAACGGCGCGUCC-5'

position 103-121
5'-UACCUGUUGCCGCGCAGGG-3'
3'-AUGGACAACGGCGCGUCCC-5'

position 127-145
5'-AGGUUGGGUGUGCGCGCGA-3'
3'-UCCAACCCACACGCGCGCU-5'

position 129-147
5'-GUUGGGUGUGCGCGCGACU-3'
3'-CAACCCACACGCGCGCUGA-5'

position 130-148
5'-UUGGGUGUGCGCGCGACUA-3'
3'-AACCCACACGCGCGCUGAU-5'

position 131-149
5'-UGGGUGUGCGCGCGACUAG-3'
3'-ACCCACACGCGCGCUGAUC-5'

position 133-151
5'-GGUGUGCGCGCGACUAGGA-3'
3'-CCACACGCGCGCUGAUCCU-5'

position 134-152
5'-GUGUGCGCGCGACUAGGAA-3'
3'-CACACGCGCGCUGAUCCUU-5'

position 135-153
5'-UGUGCGCGCGACUAGGAAG-3'

3'-ACACGCGCGCUGAUCCUUC-5'

position 136-154
5'-GUGCGCGCGACUAGGAAGA-3'
3'-CACGCGCGCUGAUCCUUCU-5'

position 137-155
5'-UGCGCGCGACUAGGAAGAC-3'
3'-ACGCGCGCUGAUCCUUCUG-5'

position 138-156
5'-GCGCGCGACUAGGAAGACU-3'
3'-CGCGCGCUGAUCCUUCUGA-5'

position 139-157
5'-CGCGCGACUAGGAAGACUU-3'
3'-GCGCGCUGAUCCUUCUGAA-5'

position 140-158
5'-GCGCGACUAGGAAGACUUC-3'
3'-CGCGCUGAUCCUUCUGAAG-5'

position 141-159
5'-CGCGACUAGGAAGACUUCC-3'
3'-GCGCUGAUCCUUCUGAAGG-5'

position 142-160
5'-GCGACUAGGAAGACUUCCG-3'
3'-CGCUGAUCCUUCUGAAGGC-5'

position 143-161
5'-CGACUAGGAAGACUUCCGA-3'
3'-GCUGAUCCUUCUGAAGGCU-5'

position 144-162
5'-GACUAGGAAGACUUCCGAG-3'
3'-CUGAUCCUUCUGAAGGCUC-5'

position 145-163
5'-ACUAGGAAGACUUCCGAGC-3'
3'-UGAUCCUUCUGAAGGCUCG-5'

position 146-164
5'-CUAGGAAGACUUCCGAGCG-3'
3'-GAUCCUUCUGAAGGCUCGC-5'

position 147-165
5'-UAGGAAGACUUCCGAGCGG-3'

3'-AUCCUUCUGAAGGCUCGCC-5'

position 148-166
5'-AGGAAGACUUCCGAGCGGU-3'
3'-UCCUUCUGAAGGCUCGCCA-5'

position 149-167
5'-GGAAGACUUCCGAGCGGUC-3'
3'-CCUUCUGAAGGCUCGCCAG-5'

position 150-168
5'-GAAGACUUCCGAGCGGUCG-3'
3'-CUUCUGAAGGCUCGCCAGC-5'

position 151-169
5'-AAGACUUCCGAGCGGUCGC-3'
3'-UUCUGAAGGCUCGCCAGCG-5'

position 152-170
5'-AGACUUCCGAGCGGUCGCA-3'
3'-UCUGAAGGCUCGCCAGCGU-5'

position 153-171
5'-GACUUCCGAGCGGUCGCAA-3'
3'-CUGAAGGCUCGCCAGCGUU-5'

position 154-172
5'-ACUUCCGAGCGGUCGCAAC-3'
3'-UGAAGGCUCGCCAGCGUUG-5'

position 155-173
5'-CUUCCGAGCGGUCGCAACC-3'
3'-GAAGGCUCGCCAGCGUUGG-5'

position 156-174
5'-UUCCGAGCGGUCGCAACCU-3'
3'-AAGGCUCGCCAGCGUUGGA-5'

position 157-175
5'-UCCGAGCGGUCGCAACCUC-3'
3'-AGGCUCGCCAGCGUUGGAG-5'

position 159-177
5'-CGAGCGGUCGCAACCUCGU-3'
3'-GCUCGCCAGCGUUGGAGCA-5'

position 160-178
5'-GAGCGGUCGCAACCUCGUG-3'

3'-CUCGCCAGCGUUGGAGCAC-5'

position 161-179
5'-AGCGGUCGCAACCUCGUGG-3'
3'-UCGCCAGCGUUGGAGCACC-5'
position 162-180
5'-GCGGUCGCAACCUCGUGGA-3'
3'-CGCCAGCGUUGGAGCACCU-5'

position 163-181
5'-CGGUCGCAACCUCGUGGAA-3'
3'-GCCAGCGUUGGAGCACCUU-5'

position 164-182
5'-GGUCGCAACCUCGUGGAAG-3'
3'-CCAGCGUUGGAGCACCUUC-5'

position 165-183
5'-GUCGCAACCUCGUGGAAGG-3'
3'-CAGCGUUGGAGCACCUUCC-5'

position 166-184
5'-UCGCAACCUCGUGGAAGGC-3'
3'-AGCGUUGGAGCACCUUCCG-5'

position 167-185
5'-CGCAACCUCGUGGAAGGCG-3'
3'-GCGUUGGAGCACCUUCCGC-5'

position 168-186
5'-GCAACCUCGUGGAAGGCGA-3'
3'-CGUUGGAGCACCUUCCGCU-5'

position 169-187
5'-CAACCUCGUGGAAGGCGAC-3'
3'-GUUGGAGCACCUUCCGCUG-5'

position 170-188
5'-AACCUCGUGGAAGGCGACA-3'
3'-UUGGAGCACCUUCCGCUGU-5'

position 171-189
5'-ACCUCGUGGAAGGCGACAA-3'
3'-UGGAGCACCUUCCGCUGUU-5'

position 172-190
5'-CCUCGUGGAAGGCGACAAC-3'
3'-GGAGCACCUUCCGCUGUUG-5'

position 173-191
5'-CUCGUGGAAGGCGACAACC-3'
3'-GAGCACCUUCCGCUGUUGG-5'
position 174-192
5'-UCGUGGAAGGCGACAACCU-3'
3'-AGCACCUUCCGCUGUUGGA-5'

position 175-193
5'-CGUGGAAGGCGACAACCUA-3'
3'-GCACCUUCCGCUGUUGGAU-5'

position 176-194
5'-GUGGAAGGCGACAACCUAU-3'
3'-CACCUUCCGCUGUUGGAUA-5'

position 177-195
5'-UGGAAGGCGACAACCUAUC-3'
3'-ACCUUCCGCUGUUGGAUAG-5'

position 178-196
5'-GGAAGGCGACAACCUAUCC-3'
3'-CCUUCCGCUGUUGGAUAGG-5'

position 179-197
5'-GAAGGCGACAACCUAUCCC-3'
3'-CUUCCGCUGUUGGAUAGGG-5'

position 180-198
5'-AAGGCGACAACCUAUCCCC-3'
3'-UUCCGCUGUUGGAUAGGGG-5'

position 181-199
5'-AGGCGACAACCUAUCCCCA-3'
3'-UCCGCUGUUGGAUAGGGGU-5'

position 182-200
5'-GGCGACAACCUAUCCCCAA-3'
3'-CCGCUGUUGGAUAGGGGUU-5'

position 183-201
5'-GCGACAACCUAUCCCCAAG-3'
3'-CGCUGUUGGAUAGGGGUUC-5'

position 184-202
5'-CGACAACCUAUCCCCAAGG-3'
3'-GCUGUUGGAUAGGGGUUCC-5'

position 185-203
5'-GACAACCUAUCCCCAAGGC-3'
3'-CUGUUGGAUAGGGGUUCCG-5'
position 186-204
5'-ACAACCUAUCCCCAAGGCU-3'
3'-UGUUGGAUAGGGGUUCCGA-5'

position 187-205
5'-CAACCUAUCCCCAAGGCUC-3'
3'-GUUGGAUAGGGGUUCCGAG-5'

position 188-206
5'-AACCUAUCCCCAAGGCUCG-3'
3'-UUGGAUAGGGGUUCCGAGC-5'

position 189-207
5'-ACCUAUCCCCAAGGCUCGC-3'
3'-UGGAUAGGGGUUCCGAGCG-5'

position 190-208
5'-CCUAUCCCCAAGGCUCGCC-3'
3'-GGAUAGGGGUUCCGAGCGG-5'

position 229-247
5'-GCUCAGCCCGGGUACCCUU-3'
3'-CGAGUCGGGCCCAUGGGAA-5'

position 230-248
5'-CUCAGCCCGGGUACCCUUG-3'
3'-GAGUCGGGCCCAUGGGAAC-5'

position 231-249
5'-UCAGCCCGGGUACCCUUGG-3'
3'-AGUCGGGCCCAUGGGAACC-5'

position 238-256
5'-GGGUACCCUUGGCCCCUCU-3'
3'-CCCAUGGGAACCGGGGAGA-5'

position 239-257
5'-GGUACCCUUGGCCCCUCUA-3'
3'-CCAUGGGAACCGGGGAGAU-5'

position 240-258
5'-GUACCCUUGGCCCCUCUAU-3'
3'-CAUGGGAACCGGGGAGAUA-5'

position 241-259
5'-UACCCUUGGCCCCUCUAUG-3'
3'-AUGGGAACCGGGGAGAUAC-5'

position 242-260
5'-ACCCUUGGCCCCUCUAUGG-3'
3'-UGGGAACCGGGGAGAUACC-5'

position 243-261
5'-CCCUUGGCCCCUCUAUGGC-3'
3'-GGGAACCGGGGAGAUACCG-5'

position 244-262
5'-CCUUGGCCCCUCUAUGGCA-3'
3'-GGAACCGGGGAGAUACCGU-5'

position 245-263
5'-CUUGGCCCCUCUAUGGCAA-3'
3'-GAACCGGGGAGAUACCGUU-5'

position 272-290
5'-UGGGGUGGGCAGGAUGGCU-3'
3'-ACCCCACCCGUCCUACCGA-5'

position 275-293
5'-GGUGGGCAGGAUGGCUCCU-3'
3'-CCACCCGUCCUACCGAGGA-5'

position 276-294
5'-GUGGGCAGGAUGGCUCCUG-3'
3'-CACCCGUCCUACCGAGGAC-5'

position 277-295
5'-UGGGCAGGAUGGCUCCUGU-3'
3'-ACCCGUCCUACCGAGGACA-5'

position 278-296
5'-GGGCAGGAUGGCUCCUGUC-3'
3'-CCCGUCCUACCGAGGACAG-5'

position 279-297
5'-GGCAGGAUGGCUCCUGUCA-3'
3'-CCGUCCUACCGAGGACAGU-5'

position 280-298
5'-GCAGGAUGGCUCCUGUCAC-3'
3'-CGUCCUACCGAGGACAGUG-5'

position 281-299
5'-CAGGAUGGCUCCUGUCACC-3'
3'-GUCCUACCGAGGACAGUGG-5'
position 282-300
5'-AGGAUGGCUCCUGUCACCC-3'
3'-UCCUACCGAGGACAGUGGG-5'

position 283-301
5'-GGAUGGCUCCUGUCACCCC-3'
3'-CCUACCGAGGACAGUGGGG-5'

position 284-302
5'-GAUGGCUCCUGUCACCCCG-3'
3'-CUACCGAGGACAGUGGGGC-5'

position 335-353
5'-CCCGGCGUAGGUCGCGUAA-3'
3'-GGGCCGCAUCCAGCGCAUU-5'

position 336-354
5'-CCGGCGUAGGUCGCGUAAU-3'
3'-GGCCGCAUCCAGCGCAUUA-5'

position 352-370
5'-AAUUUGGGUAAGGUCAUCG-3'
3'-UUAAACCCAUUCCAGUAGC-5'

position 353-371
5'-AUUUGGGUAAGGUCAUCGA-3'
3'-UAAACCCAUUCCAGUAGCU-5'

position 354-372
5'-UUUGGGUAAGGUCAUCGAU-3'
3'-AAACCCAUUCCAGUAGCUA-5'

position 355-373
5'-UUGGGUAAGGUCAUCGAUA-3'
3'-AACCCAUUCCAGUAGCUAU-5'

position 356-374
5'-UGGGUAAGGUCAUCGAUAC-3'
3'-ACCCAUUCCAGUAGCUAUG-5'

position 357-375
5'-GGGUAAGGUCAUCGAUACC-3'
3'-CCCAUUCCAGUAGCUAUGG-5'

position 358-376

5'-GGUAAGGUCAUCGAUACCC-3'
3'-CCAUUCCAGUAGCUAUGGG-5'

position 359-377
5'-GUAAGGUCAUCGAUACCCU-3'
3'-CAUUCCAGUAGCUAUGGGA-5'

position 382-400
5'-UGCGGCUUCGCCGACCUCA-3'
3'-ACGCCGAAGCGGCUGGAGU-5'

position 383-401
5'-GCGGCUUCGCCGACCUCAU-3'
3'-CGCCGAAGCGGCUGGAGUA-5'

position 384-402
5'-CGGCUUCGCCGACCUCAUG-3'
3'-GCCGAAGCGGCUGGAGUAC-5'

position 385-403
5'-GGCUUCGCCGACCUCAUGG-3'
3'-CCGAAGCGGCUGGAGUACC-5'

position 386-404
5'-GCUUCGCCGACCUCAUGGG-3'
3'-CGAAGCGGCUGGAGUACCC-5'

position 387-405
5'-CUUCGCCGACCUCAUGGGG-3'
3'-GAAGCGGCUGGAGUACCCC-5'

position 388-406
5'-UUCGCCGACCUCAUGGGGU-3'
3'-AAGCGGCUGGAGUACCCCA-5'

position 389-407
5'-UCGCCGACCUCAUGGGGUA-3'
3'-AGCGGCUGGAGUACCCCAU-5'

position 390-408
5'-CGCCGACCUCAUGGGGUAC-3'
3'-GCGGCUGGAGUACCCCAUG-5'

position 391-409
5'-GCCGACCUCAUGGGGUACA-3'
3'-CGGCUGGAGUACCCCAUGU-5'

position 392-410

5'-CCGACCUCAUGGGGUACAU-3'
3'-GGCUGGAGUACCCCAUGUA-5'

position 393-411
5'-CGACCUCAUGGGGUACAUU-3'
3'-GCUGGAGUACCCCAUGUAA-5'

position 394-412
5'-GACCUCAUGGGGUACAUUC-3'
3'-CUGGAGUACCCCAUGUAAG-5'

position 395-413
5'-ACCUCAUGGGGUACAUUCC-3'
3'-UGGAGUACCCCAUGUAAGG-5'

position 396-414
5'-CCUCAUGGGGUACAUUCCG-3'
3'-GGAGUACCCCAUGUAAGGC-5'

position 397-415
5'-CUCAUGGGGUACAUUCCGC-3'
3'-GAGUACCCCAUGUAAGGCG-5'

position 398-416
5'-UCAUGGGGUACAUUCCGCU-3'
3'-AGUACCCCAUGUAAGGCGA-5'

position 399-417
5'-CAUGGGGUACAUUCCGCUC-3'
3'-GUACCCCAUGUAAGGCGAG-5'

position 400-418
5'-AUGGGGUACAUUCCGCUCG-3'
3'-UACCCCAUGUAAGGCGAGC-5'

position 401-419
5'-UGGGGUACAUUCCGCUCGU-3'
3'-ACCCCAUGUAAGGCGAGCA-5'

position 402-420
5'-GGGGUACAUUCCGCUCGUC-3'
3'-CCCCAUGUAAGGCGAGCAG-5'

position 403-421
5'-GGGUACAUUCCGCUCGUCG-3'
3'-CCCAUGUAAGGCGAGCAGC-5'

position 404-422

5'-GGUACAUUCCGCUCGUCGG-3'
3'-CCAUGUAAGGCGAGCAGCC-5'

position 405-423
5'-GUACAUUCCGCUCGUCGGC-3'
3'-CAUGUAAGGCGAGCAGCCG-5'

position 406-424
5'-UACAUUCCGCUCGUCGGCG-3'
3'-AUGUAAGGCGAGCAGCCGC-5'

position 478-496
5'-GACGGCGUGAACUAUGCAA-3'
3'-CUGCCGCACUUGAUACGUU-5'

position 479-497
5'-ACGGCGUGAACUAUGCAAC-3'
3'-UGCCGCACUUGAUACGUUG-5'

position 480-498
5'-CGGCGUGAACUAUGCAACA-3'
3'-GCCGCACUUGAUACGUUGU-5'

position 481-499
5'-GGCGUGAACUAUGCAACAG-3'
3'-CCGCACUUGAUACGUUGUC-5'

position 482-500
5'-GCGUGAACUAUGCAACAGG-3'
3'-CGCACUUGAUACGUUGUCC-5'

position 483-501
5'-CGUGAACUAUGCAACAGGG-3'
3'-GCACUUGAUACGUUGUCCC-5'

position 484-502
5'-GUGAACUAUGCAACAGGGA-3'
3'-CACUUGAUACGUUGUCCCU-5'

position 485-503
5'-UGAACUAUGCAACAGGGAA-3'
3'-ACUUGAUACGUUGUCCCUU-5'

position 508-526
5'-CCCGGUUGCUCUUUCUCUA-3'
3'-GGGCCAACGAGAAAGAGAU-5'

position 509-527

5'-CCGGUUGCUCUUUCUCUAU-3'
3'-GGCCAACGAGAAAGAGAUA-5'
position 510-528
5'-CGGUUGCUCUUUCUCUAUC-3'
3'-GCCAACGAGAAAGAGAUAG-5'

position 511-529
5'-GGUUGCUCUUUCUCUAUCU-3'
3'-CCAACGAGAAAGAGAUAGA-5'

position 512-530
5'-GUUGCUCUUUCUCUAUCUU-3'
3'-CAACGAGAAAGAGAUAGAA-5'

position 513-531
5'-UUGCUCUUUCUCUAUCUUC-3'
3'-AACGAGAAAGAGAUAGAAG-5'

position 514-532
5'-UGCUCUUUCUCUAUCUUCC-3'
3'-ACGAGAAAGAGAUAGAAGG-5'

position 515-533
5'-GCUCUUUCUCUAUCUUCCU-3'
3'-CGAGAAAGAGAUAGAAGGA-5'

position 516-534
5'-CUCUUUCUCUAUCUUCCUC-3'
3'-GAGAAAGAGAUAGAAGGAG-5'

position 517-535
5'-UCUUUCUCUAUCUUCCUCU-3'
3'-AGAAAGAGAUAGAAGGAGA-5'

position 518-536
5'-CUUUCUCUAUCUUCCUCUU-3'
3'-GAAAGAGAUAGAAGGAGAA-5'

position 601-619
5'-UACCAUGUCACGAACGACU-3'
3'-AUGGUACAGUGCUUGCUGA-5'

position 602-620
5'-ACCAUGUCACGAACGACUG-3'
3'-UGGUACAGUGCUUGCUGAC-5'

position 604-622
5'-CAUGUCACGAACGACUGCU-3'

3'-GUACAGUGCUUGCUGACGA-5'

position 605-623
5'-AUGUCACGAACGACUGCUC-3'
3'-UACAGUGCUUGCUGACGAG-5'

position 607-625
5'-GUCACGAACGACUGCUCCA-3'
3'-CAGUGCUUGCUGACGAGGU-5'

position 608-626
5'-UCACGAACGACUGCUCCAA-3'
3'-AGUGCUUGCUGACGAGGUU-5'

position 609-627
5'-CACGAACGACUGCUCCAAC-3'
3'-GUGCUUGCUGACGAGGUUG-5'

position 955-973
5'-AUGGCUUGGGAUAUGAUGA-3'
3'-UACCGAACCCUAUACUACU-5'

position 956-974
5'-UGGCUUGGGAUAUGAUGAU-3'
3'-ACCGAACCCUAUACUACUA-5'

position 957-975
5'-GGCUUGGGAUAUGAUGAUG-3'
3'-CCGAACCCUAUACUACUAC-5'

position 958-976
5'-GCUUGGGAUAUGAUGAUGA-3'
3'-CGAACCCUAUACUACUACU-5'

position 959-977
5'-CUUGGGAUAUGAUGAUGAA-3'
3'-GAACCCUAUACUACUACUU-5'

position 961-979
5'-UGGGAUAUGAUGAUGAACU-3'
3'-ACCCUAUACUACUACUUGA-5'

position 962-980
5'-GGGAUAUGAUGAUGAACUG-3'
3'-CCCUAUACUACUACUUGAC-5'

position 963-981
5'-GGAUAUGAUGAUGAACUGG-3'

3'-CCUAUACUACUACUUGACC-5'
position 964-982
5'-GAUAUGAUGAUGAACUGGU-3'
3'-CUAUACUACUACUUGACCA-5'

position 965-983
5'-AUAUGAUGAUGAACUGGUC-3'
3'-UAUACUACUACUUGACCAG-5'

position 1072-1090
5'-GCGGGCCUUGCCUACUAUU-3'
3'-CGCCCGGAACGGAUGAUAA-5'

position 1073-1091
5'-CGGGCCUUGCCUACUAUUC-3'
3'-GCCCGGAACGGAUGAUAAG-5'

position 1074-1092
5'-GGGCCUUGCCUACUAUUCC-3'
3'-CCCGGAACGGAUGAUAAGG-5'

position 1075-1093
5'-GGCCUUGCCUACUAUUCCA-3'
3'-CCGGAACGGAUGAUAAGGU-5'

position 1076-1094
5'-GCCUUGCCUACUAUUCCAU-3'
3'-CGGAACGGAUGAUAAGGUA-5'

position 1077-1095
5'-CCUUGCCUACUAUUCCAUG-3'
3'-GGAACGGAUGAUAAGGUAC-5'

position 1078-1096
5'-CUUGCCUACUAUUCCAUGG-3'
3'-GAACGGAUGAUAAGGUACC-5'

position 1090-1108
5'-UCCAUGGUGGGGAACUGGG-3'
3'-AGGUACCACCCCUUGACCC-5'

position 1091-1109
5'-CCAUGGUGGGGAACUGGGC-3'
3'-GGUACCACCCCUUGACCCG-5'

position 3931-3949
5'-GGCAAGUUCCUUGCCGACG-3'
3'-CCGUUCAAGGAACGGCUGC-5'

position 3932-3950
5'-GCAAGUUCCUUGCCGACGG-3'
3'-CGUUCAAGGAACGGCUGCC-5'

position 3988-4006
5'-GAUGAGUGCCACUCAACUG-3'
3'-CUACUCACGGUGAGUUGAC-5'

position 3989-4007
5'-AUGAGUGCCACUCAACUGA-3'
3'-UACUCACGGUGAGUUGACU-5'

position 3990-4008
5'-UGAGUGCCACUCAACUGAC-3'
3'-ACUCACGGUGAGUUGACUG-5'

position 3991-4009
5'-GAGUGCCACUCAACUGACU-3'
3'-CUCACGGUGAGUUGACUGA-5'

position 3992-4010
5'-AGUGCCACUCAACUGACUC-3'
3'-UCACGGUGAGUUGACUGAG-5'

position 4039-4057
5'-CUGGACCAAGCGGAGACGG-3'
3'-GACCUGGUUCGCCUCUGCC-5'

position 4040-4058
5'-UGGACCAAGCGGAGACGGC-3'
3'-ACCUGGUUCGCCUCUGCCG-5'

position 4041-4059
5'-GGACCAAGCGGAGACGGCU-3'
3'-CCUGGUUCGCCUCUGCCGA-5'

position 4042-4060
5'-GACCAAGCGGAGACGGCUG-3'
3'-CUGGUUCGCCUCUGCCGAC-5'

position 4043-4061
5'-ACCAAGCGGAGACGGCUGG-3'
3'-UGGUUCGCCUCUGCCGACC-5'

position 4044-4062
5'-CCAAGCGGAGACGGCUGGA-3'
3'-GGUUCGCCUCUGCCGACCU-5'

position 4045-4063
5'-CAAGCGGAGACGGCUGGAG-3'
3'-GUUCGCCUCUGCCGACCUC-5'

position 4046-4064
5'-AAGCGGAGACGGCUGGAGC-3'
3'-UUCGCCUCUGCCGACCUCG-5'

position 4072-4090
5'-GUCGUGCUCGCCACCGCUA-3'
3'-CAGCACGAGCGGUGGCGAU-5'

position 4073-4091
5'-UCGUGCUCGCCACCGCUAC-3'
3'-AGCACGAGCGGUGGCGAUG-5'

position 4411-4429
5'-UGUGUCACCCAGACAGUCG-3'
3'-ACACAGUGGGUCUGUCAGC-5'

position 4412-4430
5'-GUGUCACCCAGACAGUCGA-3'
3'-CACAGUGGGUCUGUCAGCU-5'

position 4603-4621
5'-UAUGACGCGGGCUGUGCUU-3'
3'-AUACUGCGCCCGACACGAA-5'

position 4604-4622
5'-AUGACGCGGGCUGUGCUUG-3'
3'-UACUGCGCCCGACACGAAC-5'

position 4605-4623
5'-UGACGCGGGCUGUGCUUGG-3'
3'-ACUGCGCCCGACACGAACC-5'

position 4606-4624
5'-GACGCGGGCUGUGCUUGGU-3'
3'-CUGCGCCCGACACGAACCA-5'

position 4607-4625
5'-ACGCGGGCUGUGCUUGGUA-3'
3'-UGCGCCCGACACGAACCAU-5'

position 5323-5341
5'-UGGGCGAAGCACAUGUGGA-3'
3'-ACCCGCUUCGUGUACACCU-5'

position 5324-5342
5'-GGGCGAAGCACAUGUGGAA-3'
3'-CCCGCUUCGUGUACACCUU-5'

position 5776-5794
5'-GCUGUGCAGUGGAUGAACC-3'
3'-CGACACGUCACCUACUUGG-5'

position 5777-5795
5'-CUGUGCAGUGGAUGAACCG-3'
3'-GACACGUCACCUACUUGGC-5'

position 5778-5796
5'-UGUGCAGUGGAUGAACCGG-3'
3'-ACACGUCACCUACUUGGCC-5'

position 5779-5797
5'-GUGCAGUGGAUGAACCGGC-3'
3'-CACGUCACCUACUUGGCCG-5'

position 5780-5798
5'-UGCAGUGGAUGAACCGGCU-3'
3'-ACGUCACCUACUUGGCCGA-5'

position 5781-5799
5'-GCAGUGGAUGAACCGGCUG-3'
3'-CGUCACCUACUUGGCCGAC-5'

position 5782-5800
5'-CAGUGGAUGAACCGGCUGA-3'
3'-GUCACCUACUUGGCCGACU-5'

position 5783-5801
5'-AGUGGAUGAACCGGCUGAU-3'
3'-UCACCUACUUGGCCGACUA-5'

position 5784-5802
5'-GUGGAUGAACCGGCUGAUA-3'
3'-CACCUACUUGGCCGACUAU-5'

position 5785-5803
5'-UGGAUGAACCGGCUGAUAG-3'
3'-ACCUACUUGGCCGACUAUC-5'

position 5786-5804
5'-GGAUGAACCGGCUGAUAGC-3'
3'-CCUACUUGGCCGACUAUCG-5'

position 5787-5805
5'-GAUGAACCGGCUGAUAGCG-3'
3'-CUACUUGGCCGACUAUCGC-5'

position 5788-5806
5'-AUGAACCGGCUGAUAGCGU-3'
3'-UACUUGGCCGACUAUCGCA-5'

position 5789-5807
5'-UGAACCGGCUGAUAGCGUU-3'
3'-ACUUGGCCGACUAUCGCAA-5'

position 5790-5808
5'-GAACCGGCUGAUAGCGUUC-3'
3'-CUUGGCCGACUAUCGCAAG-5'

position 5791-5809
5'-AACCGGCUGAUAGCGUUCG-3'
3'-UUGGCCGACUAUCGCAAGC-5'

position 5792-5810
5'-ACCGGCUGAUAGCGUUCGC-3'
3'-UGGCCGACUAUCGCAAGCG-5'

position 6013-6031
5'-UUCAAGACCUGGCUCCAGU-3'
3'-AAGUUCUGGACCGAGGUCA-5'

position 6014-6032
5'-UCAAGACCUGGCUCCAGUC-3'
3'-AGUUCUGGACCGAGGUCAG-5'

position 6016-6034
5'-AAGACCUGGCUCCAGUCCA-3'
3'-UUCUGGACCGAGGUCAGGU-5'

position 6017-6035
5'-AGACCUGGCUCCAGUCCAA-3'
3'-UCUGGACCGAGGUCAGGUU-5'

position 6019-6037
5'-ACCUGGCUCCAGUCCAAGC-3'
3'-UGGACCGAGGUCAGGUUCG-5'

position 6020-6038
5'-CCUGGCUCCAGUCCAAGCU-3'
3'-GGACCGAGGUCAGGUUCGA-5'

position 6329-6347
5'-UGGGGGAUUUCCACUACGU-3'
3'-ACCCCCUAAAGGUGAUGCA-5'

position 6331-6349
5'-GGGGAUUUCCACUACGUGA-3'
3'-CCCCUAAAGGUGAUGCACU-5'

position 6349-6367
5'-ACGGGCAUGACCACUGACA-3'
3'-UGCCCGUACUGGUGACUGU-5'

position 6350-6368
5'-CGGGCAUGACCACUGACAA-3'
3'-GCCCGUACUGGUGACUGUU-5'

position 6351-6369
5'-GGGCAUGACCACUGACAAC-3'
3'-CCCGUACUGGUGACUGUUG-5'

position 6481-6499
5'-UUCCAGGUCGGGCUCAACC-3'
3'-AAGGUCCAGCCCGAGUUGG-5'

position 6482-6500
5'-UCCAGGUCGGGCUCAACCA-3'
3'-AGGUCCAGCCCGAGUUGGU-5'

position 6755-6773
5'-UGUGGCGGCAGGAGAUGGG-3'
3'-ACACCGCCGUCCUCUACCC-5'

position 7585-7603
5'-GUCCUGGACGACCACUACC-3'
3'-CAGGACCUGCUGGUGAUGG-5'

position 7586-7604
5'-UCCUGGACGACCACUACCG-3'
3'-AGGACCUGCUGGUGAUGGC-5'

position 7587-7605
5'-CCUGGACGACCACUACCGG-3'
3'-GGACCUGCUGGUGAUGGCC-5'

position 7588-7606
5'-CUGGACGACCACUACCGGG-3'
3'-GACCUGCUGGUGAUGGCCC-5'

position 7590-7608
5'-GGACGACCACUACCGGGAC-3'
3'-CCUGCUGGUGAUGGCCCUG-5'

position 7591-7609
5'-GACGACCACUACCGGGACG-3'
3'-CUGCUGGUGAUGGCCCUGC-5'

position 7592-7610
5'-ACGACCACUACCGGGACGU-3'
3'-UGCUGGUGAUGGCCCUGCA-5'

position 7600-7618
5'-UACCGGGACGUGCUCAAGG-3'
3'-AUGGCCCUGCACGAGUUCC-5'

position 7601-7619
5'-ACCGGGACGUGCUCAAGGA-3'
3'-UGGCCCUGCACGAGUUCCU-5'

position 7602-7620
5'-CCGGGACGUGCUCAAGGAG-3'
3'-GGCCCUGCACGAGUUCCUC-5'

position 7612-7630
5'-CUCAAGGAGAUGAAGGCGA-3'
3'-GAGUUCCUCUACUUCCGCU-5'

position 7613-7631
5'-UCAAGGAGAUGAAGGCGAA-3'
3'-AGUUCCUCUACUUCCGCUU-5'

position 7614-7632
5'-CAAGGAGAUGAAGGCGAAG-3'
3'-GUUCCUCUACUUCCGCUUC-5'

position 7615-7633
5'-AAGGAGAUGAAGGCGAAGG-3'
3'-UUCCUCUACUUCCGCUUCC-5'

position 7616-7634
5'-AGGAGAUGAAGGCGAAGGC-3'
3'-UCCUCUACUUCCGCUUCCG-5'

position 7617-7635
5'-GGAGAUGAAGGCGAAGGCG-3'
3'-CCUCUACUUCCGCUUCCGC-5'

position 7618-7636
5'-GAGAUGAAGGCGAAGGCGU-3'
3'-CUCUACUUCCGCUUCCGCA-5'

position 7619-7637
5'-AGAUGAAGGCGAAGGCGUC-3'
3'-UCUACUUCCGCUUCCGCAG-5'

position 7620-7638
5'-GAUGAAGGCGAAGGCGUCC-3'
3'-CUACUUCCGCUUCCGCAGG-5'

position 7621-7639
5'-AUGAAGGCGAAGGCGUCCA-3'
3'-UACUUCCGCUUCCGCAGGU-5'

position 7622-7640
5'-UGAAGGCGAAGGCGUCCAC-3'
3'-ACUUCCGCUUCCGCAGGUG-5'

position 7623-7641
5'-GAAGGCGAAGGCGUCCACA-3'
3'-CUUCCGCUUCCGCAGGUGU-5'

position 7624-7642
5'-AAGGCGAAGGCGUCCACAG-3'
3'-UUCCGCUUCCGCAGGUGUC-5'

position 7625-7643
5'-AGGCGAAGGCGUCCACAGU-3'
3'-UCCGCUUCCGCAGGUGUCA-5'

position 8135-8153
5'-GUUGUGACUUGGCCCCCGA-3'
3'-CAACACUGAACCGGGGGCU-5'

position 8337-8355
5'-GGACUGCACGAUGCUCGUG-3'
3'-CCUGACGUGCUACGAGCAC-5'

position 8415-8433
5'-CCUACGAGUCUUCACGGAG-3'
3'-GGAUGCUCAGAAGUGCCUC-5'

position 8416-8434
5'-CUACGAGUCUUCACGGAGG-3'
3'-GAUGCUCAGAAGUGCCUCC-5'

position 8417-8435
5'-UACGAGUCUUCACGGAGGC-3'
3'-AUGCUCAGAAGUGCCUCCG-5'

position 8418-8436
5'-ACGAGUCUUCACGGAGGCU-3'
3'-UGCUCAGAAGUGCCUCCGA-5'

position 8419-8437
5'-CGAGUCUUCACGGAGGCUA-3'
3'-GCUCAGAAGUGCCUCCGAU-5'

position 8420-8438
5'-GAGUCUUCACGGAGGCUAU-3'
3'-CUCAGAAGUGCCUCCGAUA-5'

position 8421-8439
5'-AGUCUUCACGGAGGCUAUG-3'
3'-UCAGAAGUGCCUCCGAUAC-5'

position 8422-8440
5'-GUCUUCACGGAGGCUAUGA-3'
3'-CAGAAGUGCCUCCGAUACU-5'

position 8423-8441
5'-UCUUCACGGAGGCUAUGAC-3'
3'-AGAAGUGCCUCCGAUACUG-5'

position 8424-8442
5'-CUUCACGGAGGCUAUGACU-3'
3'-GAAGUGCCUCCGAUACUGA-5'

position 8425-8443
5'-UUCACGGAGGCUAUGACUA-3'
3'-AAGUGCCUCCGAUACUGAU-5'

position 8426-8444
5'-UCACGGAGGCUAUGACUAG-3'
3'-AGUGCCUCCGAUACUGAUC-5'

position 8427-8445
5'-CACGGAGGCUAUGACUAGG-3'
3'-GUGCCUCCGAUACUGAUCC-5'

position 8428-8446
5'-ACGGAGGCUAUGACUAGGU-3'
3'-UGCCUCCGAUACUGAUCCA-5'
position 8429-8447
5'-CGGAGGCUAUGACUAGGUA-3'
3'-GCCUCCGAUACUGAUCCAU-5'

position 8430-8448
5'-GGAGGCUAUGACUAGGUAC-3'
3'-CCUCCGAUACUGAUCCAUG-5'

position 8431-8449
5'-GAGGCUAUGACUAGGUACU-3'
3'-CUCCGAUACUGAUCCAUGA-5'

position 8432-8450
5'-AGGCUAUGACUAGGUACUC-3'
3'-UCCGAUACUGAUCCAUGAG-5'

position 8626-8644
5'-AACUCCUGGCUAGGCAACA-3'
3'-UUGAGGACCGAUCCGUUGU-5'

position 8627-8645
5'-ACUCCUGGCUAGGCAACAU-3'
3'-UGAGGACCGAUCCGUUGUA-5'

position 8776-8794
5'-CCACUUGACCUACCUCAGA-3'
3'-GGUGAACUGGAUGGAGUCU-5'

position 8777-8795
5'-CACUUGACCUACCUCAGAU-3'
3'-GUGAACUGGAUGGAGUCUA-5'

position 8778-8796
5'-ACUUGACCUACCUCAGAUC-3'
3'-UGAACUGGAUGGAGUCUAG-5'

position 8779-8797
5'-CUUGACCUACCUCAGAUCA-3'
3'-GAACUGGAUGGAGUCUAGU-5'

position 8780-8798
5'-UUGACCUACCUCAGAUCAU-3'
3'-AACUGGAUGGAGUCUAGUA-5'

position 8781-8799

5'-UGACCUACCUCAGAUCAUU-3'
3'-ACUGGAUGGAGUCUAGUAA-5'

position 8803-8821
5'-CGACUCCAUGGUCUUAGCG-3'
3'-GCUGAGGUACCAGAAUCGC-5'

position 8804-8822
5'-GACUCCAUGGUCUUAGCGC-3'
3'-CUGAGGUACCAGAAUCGCG-5'

position 8815-8833
5'-CUUAGCGCAUUUUCACUCC-3'
3'-GAAUCGCGUAAAAGUGAGG-5'

position 8816-8834
5'-UUAGCGCAUUUUCACUCCA-3'
3'-AAUCGCGUAAAAGUGAGGU-5'

position 8836-8854
5'-AGUUACUCUCCAGGUGAGA-3'
3'-UCAAUGAGAGGUCCACUCU-5'

position 8837-8855
5'-GUUACUCUCCAGGUGAGAU-3'
3'-CAAUGAGAGGUCCACUCUA-5'

position 8838-8856
5'-UUACUCUCCAGGUGAGAUC-3'
3'-AAUGAGAGGUCCACUCUAG-5'

position 8839-8857
5'-UACUCUCCAGGUGAGAUCA-3'
3'-AUGAGAGGUCCACUCUAGU-5'

position 8840-8858
5'-ACUCUCCAGGUGAGAUCAA-3'
3'-UGAGAGGUCCACUCUAGUU-5'

position 8841-8859
5'-CUCUCCAGGUGAGAUCAAU-3'
3'-GAGAGGUCCACUCUAGUUA-5'

position 8842-8860
5'-UCUCCAGGUGAGAUCAAUA-3'
3'-AGAGGUCCACUCUAGUUAU-5'

position 8843-8861

5'-CUCCAGGUGAGAUCAAUAG-3'
3'-GAGGUCCACUCUAGUUAUC-5'

position 8844-8862
5'-UCCAGGUGAGAUCAAUAGG-3'
3'-AGGUCCACUCUAGUUAUCC-5'

position 8845-8863
5'-CCAGGUGAGAUCAAUAGGG-3'
3'-GGUCCACUCUAGUUAUCCC-5'

position 8846-8864
5'-CAGGUGAGAUCAAUAGGGU-3'
3'-GUCCACUCUAGUUAUCCCA-5'

position 8847-8865
5'-AGGUGAGAUCAAUAGGGUG-3'
3'-UCCACUCUAGUUAUCCCAC-5'

position 8848-8866
5'-GGUGAGAUCAAUAGGGUGG-3'
3'-CCACUCUAGUUAUCCCACC-5'

position 8849-8867
5'-GUGAGAUCAAUAGGGUGGC-3'
3'-CACUCUAGUUAUCCCACCG-5'

position 8851-8869
5'-GAGAUCAAUAGGGUGGCUU-3'
3'-CUCUAGUUAUCCCACCGAA-5'

position 8852-8870
5'-AGAUCAAUAGGGUGGCUUC-3'
3'-UCUAGUUAUCCCACCGAAG-5'

position 8854-8872
5'-AUCAAUAGGGUGGCUUCAU-3'
3'-UAGUUAUCCCACCGAAGUA-5'

position 8855-8873
5'-UCAAUAGGGUGGCUUCAUG-3'
3'-AGUUAUCCCACCGAAGUAC-5'

position 8856-8874
5'-CAAUAGGGUGGCUUCAUGC-3'
3'-GUUAUCCCACCGAAGUACG-5'

position 8857-8875

5'-AAUAGGGUGGCUUCAUGCC-3'
3'-UUAUCCCACCGAAGUACGG-5'

position 8858-8876
5'-AUAGGGUGGCUUCAUGCCU-3'
3'-UAUCCCACCGAAGUACGGA-5'

position 8859-8877
5'-UAGGGUGGCUUCAUGCCUC-3'
3'-AUCCCACCGAAGUACGGAG-5'

position 8860-8878
5'-AGGGUGGCUUCAUGCCUCA-3'
3'-UCCCACCGAAGUACGGAGU-5'

position 8861-8879
5'-GGGUGGCUUCAUGCCUCAG-3'
3'-CCCACCGAAGUACGGAGUC-5'

position 8862-8880
5'-GGUGGCUUCAUGCCUCAGG-3'
3'-CCACCGAAGUACGGAGUCC-5'

position 8863-8881
5'-GUGGCUUCAUGCCUCAGGA-3'
3'-CACCGAAGUACGGAGUCCU-5'

position 8864-8882
5'-UGGCUUCAUGCCUCAGGAA-3'
3'-ACCGAAGUACGGAGUCCUU-5'

position 8869-8887
5'-UCAUGCCUCAGGAAACUUG-3'
3'-AGUACGGAGUCCUUUGAAC-5'

position 8870-8888
5'-CAUGCCUCAGGAAACUUGG-3'
3'-GUACGGAGUCCUUUGAACC-5'

position 8871-8889
5'-AUGCCUCAGGAAACUUGGG-3'
3'-UACGGAGUCCUUUGAACCC-5'

position 8872-8890
5'-UGCCUCAGGAAACUUGGGG-3'
3'-ACGGAGUCCUUUGAACCCC-5'

position 8873-8891

5'-GCCUCAGGAAACUUGGGGU-3'
3'-CGGAGUCCUUUGAACCCCA-5'

position 8900-8918
5'-UGCGAGUCUGGAGACAUCG-3'
3'-ACGCUCAGACCUCUGUAGC-5'

position 8901-8919
5'-GCGAGUCUGGAGACAUCGG-3'
3'-CGCUCAGACCUCUGUAGCC-5'

position 8902-8920
5'-CGAGUCUGGAGACAUCGGG-3'
3'-GCUCAGACCUCUGUAGCCC-5'

position 8903-8921
5'-GAGUCUGGAGACAUCGGGC-3'
3'-CUCAGACCUCUGUAGCCCG-5'

position 8904-8922
5'-AGUCUGGAGACAUCGGGCC-3'
3'-UCAGACCUCUGUAGCCCGG-5'

position 8905-8923
5'-GUCUGGAGACAUCGGGCCA-3'
3'-CAGACCUCUGUAGCCCGGU-5'

position 8906-8924
5'-UCUGGAGACAUCGGGCCAG-3'
3'-AGACCUCUGUAGCCCGGUC-5'

position 8907-8925
5'-CUGGAGACAUCGGGCCAGA-3'
3'-GACCUCUGUAGCCCGGUCU-5'

position 8908-8926
5'-UGGAGACAUCGGGCCAGAA-3'
3'-ACCUCUGUAGCCCGGUCUU-5'

position 8909-8927
5'-GGAGACAUCGGGCCAGAAG-3'
3'-CCUCUGUAGCCCGGUCUUC-5'

position 8910-8928
5'-GAGACAUCGGGCCAGAAGU-3'
3'-CUCUGUAGCCCGGUCUUCA-5'

position 8911-8929
5'-AGACAUCGGGCCAGAAGUG-3'
3'-UCUGUAGCCCGGUCUUCAC-5'

position 8912-8930
5'-GACAUCGGGCCAGAAGUGU-3'
3'-CUGUAGCCCGGUCUUCACA-5'

position 8980-8998
5'-UACCUCUUCAACUGGGCAG-3'
3'-AUGGAGAAGUUGACCCGUC-5'

position 8981-8999
5'-ACCUCUUCAACUGGGCAGU-3'
3'-UGGAGAAGUUGACCCGUCA-5'

position 8982-9000
5'-CCUCUUCAACUGGGCAGUA-3'
3'-GGAGAAGUUGACCCGUCAU-5'

position 8983-9001
5'-CUCUUCAACUGGGCAGUAA-3'
3'-GAGAAGUUGACCCGUCAUU-5'

position 9070-9088
5'-GGUUACAGCGGGGGAGACA-3'
3'-CCAAUGUCGCCCCCUCUGU-5'

position 9073-9091
5'-UACAGCGGGGGAGACAUAU-3'
3'-AUGUCGCCCCCUCUGUAUA-5'

position 9074-9092
5'-ACAGCGGGGGAGACAUAUA-3'
3'-UGUCGCCCCCUCUGUAUAU-5'

position 9075-9093
5'-CAGCGGGGGAGACAUAUAU-3'
3'-GUCGCCCCCUCUGUAUAUA-5'

position 9076-9094
5'-AGCGGGGGAGACAUAUAUC-3'
3'-UCGCCCCCUCUGUAUAUAG-5'

position 9077-9095
5'-GCGGGGGAGACAUAUAUCA-3'
3'-CGCCCCCUCUGUAUAUAGU-5'

position 9078-9096
5'-CGGGGGAGACAUAUAUCAC-3'
3'-GCCCCCUCUGUAUAUAGUG-5'

position 9079-9097
5'-GGGGGAGACAUAUAUCACA-3'
3'-CCCCCUCUGUAUAUAGUGU-5'

position 9080-9098
5'-GGGGAGACAUAUAUCACAG-3'
3'-CCCCUCUGUAUAUAGUGUC-5'

position 9081-9099
5'-GGGAGACAUAUAUCACAGC-3'
3'-CCCUCUGUAUAUAGUGUCG-5'

position 9082-9100
5'-GGAGACAUAUAUCACAGCC-3'
3'-CCUCUGUAUAUAGUGUCGG-5'

position 9083-9101
5'-GAGACAUAUAUCACAGCCU-3'
3'-CUCUGUAUAUAGUGUCGGA-5'

position 9084-9102
5'-AGACAUAUAUCACAGCCUG-3'
3'-UCUGUAUAUAGUGUCGGAC-5'

position 9085-9103
5'-GACAUAUAUCACAGCCUGU-3'
3'-CUGUAUAUAGUGUCGGACA-5'

position 9086-9104
5'-ACAUAUAUCACAGCCUGUC-3'
3'-UGUAUAUAGUGUCGGACAG-5'

position 9087-9105
5'-CAUAUAUCACAGCCUGUCU-3'
3'-GUAUAUAGUGUCGGACAGA-5'

position 9088-9106
5'-AUAUAUCACAGCCUGUCUC-3'
3'-UAUAUAGUGUCGGACAGAG-5'

The following dsRNA oligonucleotide pairs (designated LZ1-LZ150) that are complementary (homologous) in sequence to various regions of HCV structural and nonstructural genes have also been identified as novel anti-HCV agents for use in

silencing HCV gene expression or otherwise preventing or treating HCV infections. While shown comprising two 2'-deoxythymidine overhangs at the 3' end of each strand, two uridine residues can be present instead. These, as well as the foregoing and other dsRNA oligonucleotides disclosed herein, can be obtained from suppliers such as Dharmacon Research, Inc., Boulder, CO.:

LZ-PAIR-1: 5'-GGGCGACACUCCACCAUAGdTdT-3'
3'-dTdTCCCGCUGUGAGGUGGUAUC-5' ;

LZ-PAIR-2: 5'-GGACCCCCCCUCCCGGGAGdTdT-3'
3'-dTdTCCUGGGGGGGAGGGCCCTC-5' ;

LZ-PAIR-3: 5'-GCCUGGAGAUUUGGGCGTGdTdT-3'
3'-dTdTCGGACCUCUAAACCCGCAC-5' ;

LZ-PAIR-4: 5'-CGGGAGGUCUCGUAGACCGdTdT-3'
3'-dTdTGCCCUCCAGAGCAUCUGGC-5' ;

LZ-PAIR-5: 5'-GGCGGUGGUCAGAUCGUCGdTdT-3'
3'-dTdTCCGCCACCAGUCUAGCAGC-5' ;

LZ-PAIR-6: 5'-GGAAGGCGACAACCUAUCCdTdT-3'
3'-dTdTCCUUCCGCUGUUGGAUAGG-5' ;

LZ-PAIR-7: 5'-GGGCAGGAUGGCUCCUGUCdTdT-3'
3'-dTdTCCCGUCCUACCGAGGACAG-5' ;

LZ-PAIR-8: 5'-CCUCACGUGCGGCUUCGCCdTdT-3'
3'-dTdTGGAGUGCACGCCGAAGCGG-5' ;

LZ-PAIR-9: 5'-GAGGACGGCGUGAACUAUGdTdT-3'
3'-dTdTCUCCUGCCGCACUUGAUAC-5' ;

LZ-PAIR-10: 5'-GAAGUGCGCAACGUAUCCGdTdT-3'
3'-dTdTCUUCACGCGUUGCAUAGGC-5' ;

LZ-PAIR-11: 5'-GUGCGUGCCCUGCGUUCGGdTdT-3'
3'-dTdTCACGCACGGGACGCAAGCC-5' ;

LZ-PAIR-12: 5'-CGACGCCAUGUCGAUUUGCdTdT-3'
3'-dTdTGCUGCGGUACAGCUAAACG-5' ;

LZ-PAIR-13: 5'-CUCGCCUCGCCGGCACGAGdTdT-3'
3'-dTdTGAGCGGAGCGGCCGUGCUC-5' ;

LZ-PAIR-14: 5'-CCUACAGCAGCCCUAGUGGdTdT-3'
3'-dTdTGGAUGUCGUCGGGAUCACC-5' ;

LZ-PAIR-15: 5'-CUACUAUUCCAUGGUGGGGdTdT-3'
3'-dTdTGAUGAUAAGGUACCACCCC-5' ;

LZ-PAIR-16: 5'-CACCCUCGGGAUUACGUCCdTdT-3'
3'-dTdTGUGGGAGCCCUAAUGCAGG-5' ;

LZ-PAIR-17: 5'-GAACUGCAAUGACUCCCUCdTdT-3'
3'-dTdTCUUGACGUUACUGAGGGAG-5' ;

LZ-PAIR-18: 5'-GCCCCAUCGACGCGUUCGCdTdT-3'
3'-dTdTCGGGGUAGCUGCGCAAGCG-5' ;

LZ-PAIR-19: 5'-GCGGUAUCGUACCCGCGGCdTdT-3'
3'-dTdTCGCCAUAGCAUGGGCGCCG-5' ;

LZ-PAIR-20: 5'-CAGUUGGGGGGAGAAUGAGdTdT-3'
3'-dTdTGUCAACCCCCCUCUUACUC-5' ;

LZ-PAIR-21: 5'-CCAAGACGUGCGGGGGCCCdTdT-3'
3'-dTdTGGUUCUGCACGCCCCCGGG-5' ;

LZ-PAIR-22: 5'-CACCAAGUGUGGUUCGGGGdTdT-3'
3'-dTdTGUGGUUCACACCAAGCCCC-5' ;

LZ-PAIR-23: 5'-CAAGGUUAGGAUGUACGUGdTdT-3'
3'-dTdTGUUCCAAUCCUACAUGCAC-5' ;

LZ-PAIR-24: 5'-GAGCUUAGCCCGCUGCUGCdTdT-3'
3'-dTdTCUCGAAUCGGGCGACGACG-5' ;

LZ-PAIR-25: 5'-CAGAACGUCGUGGACGUACdTdT-3'
3'-dTdTGUCUUGCAGCACCUGCAUG-5' ;

LZ-PAIR-26: 5'-GGACGCGCGCGUCUGUGCCdTdT-3'
3'-dTdTCCUGCGCGCGCAGACACGG-5' ;

LZ-PAIR-27: 5'-CGGGGCGCAUGGCAUUCUCdTdT-3'
3'-dTdTGCCCCGCGUACCGUAAGAG-5' ;

LZ-PAIR-28: 5'-CCGCUACUCCUGCUCCUGCdTdT-3'
3'-dTdTGGCGAUGAGGACGAGGACG-5' ;

LZ-PAIR-29: 5'-CUCUUGACCUUGUCACCGCdTdT-3'
3'-dTdTGAGAACUGGAACAGUGGCG-5' ;

LZ-PAIR-30: 5'-CCCCCCCCUCAACGUUCGGdTdT-3'
3'-dTdTGGGGGGGGAGUUGCAAGCC-5' ;

LZ-PAIR-31: 5'-CGCCAUACUCGGUCCACUCdTdT-3'
3'-dTdTGCGGUAUGAGCCAGGUGAG-5' ;

LZ-PAIR-32: 5'-CGGAAGGUUGCUGGGGGUCdTdT-3'
3'-dTdTGCCUUCCAACGACCCCCAG-5' ;

LZ-PAIR-33: 5'-CCACGCGGGCCUACGAGACdTdT-3'
3'-dTdTGGUGCGCCCGGAUGCUCUG-5' ;

LZ-PAIR-34: 5'-GGGGACAUCAUCUUGGGCCdTdT-3'
3'-dTdTCCCCUGUAGUAGAACCCGG-5' ;

LZ-PAIR-35: 5'-CGCCUAUUACGGCCUACUCdTdT-3'
3'-dTdTGCGGAUAAUGCCGGAUGAG-5' ;

LZ-PAIR-36: 5'-GUGGUCUCCACCGCAACACdTdT-3'
3'-dTdTCACCAGAGGUGGCGUUGUG-5' ;

LZ-PAIR-37: 5'-GGGCCCAAUCACCCAAAUGdTdT-3'
3'-dTdTCCCGGGUUAGUGGGUUUAC-5' ;

LZ-PAIR-38: 5'-CUCGGACCUUUACUUGGUCdTdT-3'
3'-dTdTGAGCCUGGAAAUGAACCAG-5' ;

LZ-PAIR-39: 5'-CUUGAAGGGCUCUUCGGGCdTdT-3'
3'-dTdTGAACUUCCCGAGAAGCCCGdTdT-3'

LZ-PAIR-40: 5'-GGACUUUGUACCCGUCGAGdTdT-3'
3'-dTdTCCUGAAACAUGGGCAGCUC-5' ;

LZ-PAIR-41: 5'-CAUCUACACGCCCCUACUGdTdT-3'
3'-dTdTGUAGAUGUGCGGGGAUGAC-5' ;

LZ-PAIR-42: 5'-CACCCUAGGUUUCGGGGCGdTdT-3'
3'-dTdTGUGGGAUCCAAAGCCCCGC-5' ;

LZ-PAIR-43: 5'-CCUAUGGCAAGUUUCUUGCdTdT-3'
3'-dTdTGGAUACCGUUCAAAGAACG-5' ;

LZ-PAIR-44: 5'-GGGCAUCGGCACAGUCCUGdTdT-3'
3'-dTdTCCCGUAGCCGUGUCAGGAC-5' ;

LZ-PAIR-45: 5'-CAAACAUCGAGGAGGUGGCdTdT-3'
3'-dTdTGUUUGUAGCUCCUCCACCG-5' ;

LZ-PAIR-46: 5'-GCCAUUCCAAGAAGAAAUGdTdT-3'
3'-dTdTCGGUAAGGUUCUUCUUUAC-5' ;

LZ-PAIR-47: 5'-CCGUCAUACCAACUAGCGGdTdT-3'
3'-dTdTGGCAGUAUGGUUGAUCGCC-5' ;

LZ-PAIR-48: 5'-GACAGUCGACUUCAGCCUGdTdT-3'
3'-dTdTCUGUCAGCUGAAGUCGGAC-5' ;

LZ-PAIR-49: 5'-GCAGGAUGGGCAUUUACAGdTdT-3'
3'-dTdTCGUCCUACCCGUAAAUGUC-5' ;

LZ-PAIR-50: 5'-GGUACGAGCUCACGCCCGCdTdT-3'
3'-dTdTCCAUGCUCGAGUGCGGGCG-5' ;

LZ-PAIR-51: 5'-CGUCUUUACAGGCCUCACCdTdT-3'
3'-dTdTGCAGAAAUGUCCGGAGUGG-5' ;

LZ-PAIR-52: 5'-GCGCCAGGGCUCAGGCUCCdTdT-3'
3'-dTdTCGCGGUCCCGAGUCCGAGG-5' ;

LZ-PAIR-53: 5'-GCUGGGAGCCGUUCAAAACdTdT-3'
3'-dTdTCGACCCUCGGCAAGUUUUG-5' ;

LZ-PAIR-54: 5'-GUGCUGGUAGGCGGAGUCCdTdT-3'
3'-dTdTCACGACCAUCCGCCUCAGG-5' ;

LZ-PAIR-55: 5'-CAUUCCCGACAGGGAAGUCdTdT-3'
3'-dTdTGUAAGGGCUGUCCCUUCAG-5' ;

LZ-PAIR-56: 5'-CUCGCCGAACAAUUCAAACdTdT-3'
3'-dTdTGAGCGGCUUGUUAAGUUUG-5' ;

LZ-PAIR-57: 5'-CUGGGCGAAGCAUAUGUGGdTdT-3'
3'-dTdTGACCCGCUUCGUAUACACC-5' ;

LZ-PAIR-58: 5'-CAGCCUCUAUCACCAGCCCdTdT-3'
3'-dTdTGUCGGAGAUAGUGGUCGGG-5' ;

LZ-PAIR-59: 5'-CUGCUUUCGUAGGCGCCGGdTdT-3'
3'-dTdTGACGAAAGCAUCCGCGGCC-5' ;

LZ-PAIR-60: 5'-GGCAGGCGCGCUCGUGGCCdTdT-3'
3'-dTdTCCGUCCGCGCGAGCACCGG-5' ;

LZ-PAIR-61: 5'-GUCGGGGUCGUGUGCGCAGdTdT-3'
3'-dTdTCAGCCCCAGCACACGCGTC-5' ;

LZ-PAIR-62: 5'-CCACGUCUCCCCCACGCACdTdT-3'
3'-dTdTGGUGCAGAGGGGGUGCGUG-5' ;

LZ-PAIR-63: 5'-CACCAGUGGAUCAACGAGGdTdT-3'
3'-dTdTGUGGUCACCUAGUUGCUCC-5' ;

LZ-PAIR-64: 5'-CUGGCUCCAGUCCAAGCUCdTdT-3'
3'-dTdTGACCGAGGUCAGGUUCGAG-5' ;

LZ-PAIR-65: 5'-CCACCUGCCCAUGUGGAGCdTdT-3'
3'-dTdTGGUGGACGGGUACACCUCG-5' ;

LZ-PAIR-66: 5'-CCCCAUUAACGCGUACACCdTdT-3'
3'-dTdTGGGGUAAUUGCGCAUGUGG-5' ;

LZ-PAIR-67: 5'-GUGGGGGAUUUCCACUACGdTdT-3'
3'-dTdTCACCCCCUAAAGGUGAUGC-5' ;

LZ-PAIR-68: 5'-GUGCGGUUGCACAGGUACGdTdT-3'
3'-dTdTCACGCCAACGUGUCCAUGC-5' ;

LZ-PAIR-69: 5'-CCCAUGCGAGCCCGAACCGdTdT-3'
3'-dTdTGGGUACGCUCGGGCUUGGC-5' ;

LZ-PAIR-70: 5'-CUCCCCCCUCCUUGGCCAGdTdT-3'
3'-dTdTGAGGGGGGAGGAACCGGUC-5' ;

LZ-PAIR-71: 5'-CGAGGCCAACCUCCUGUGGdTdT-3'
3'-dTdTGCUCCGGUUGGAGGACACC-5' ;

LZ-PAIR-72: 5'-GCGGAGGAGGAUGAGAGGGdTdT-3'
3'-dTdTCGCCUCCUCCUACUCUCCC-5' ;

LZ-PAIR-73: 5'-CCCUCCACUGUUAGAGUCCdTdT-3'
3'-dTdTGGGAGGUGACAAUCUCAGG-5' ;

LZ-PAIR-74: 5'-CGGAGGAAGAGGACGGUUGdTdT-3'
3'-dTdTGCCUCCUUCUCCUGCCAAC-5' ;

LZ-PAIR-75: 5'-GACAGCGGCACGGCAACGGdTdT-3'
3'-dTdTCUGUCGCCGUGCCGUUGCC-5' ;

LZ-PAIR-76: 5'-GCCGGGGGAUCCCGAUCUCdTdT-3'
3'-dTdTCGGCCCCCUAGGGCUAGAG-5' ;

LZ-PAIR-77: 5'-CGCCCUGAUCACGCCAUGCdTdT-3'
3'-dTdTGCGGGACUAGUGCGGUACG-5' ;

LZ-PAIR-78: 5'-CUCGCAGCGCAAGCCUGCGdTdT-3'
3'-dTdTGAGCGUCGCGUUCGGACGC-5' ;

LZ-PAIR-79: 5'-GAAGGCGUCCACAGUUAAGdTdT-3'
3'-dTdTCUUCCGCAGGUGUCAAUUC-5' ;

LZ-PAIR-80: 5'-CGUCCGGAACCUAUCCAGCdTdT-3'
3'-dTdTGCAGGCCUUGGAUAGGUCG-5' ;

LZ-PAIR-81: 5'-GAGGUUUUCUGCGUCCAACdTdT-3'
3'-dTdTCUCCAAAAGACGCAGGUUG-5' ;

LZ-PAIR-82: 5'-GGCCCUUUACGAUGUGGUCdTdT-3'
3'-dTdTCCGGGAAAUGCUACACCAG-5' ;

LZ-PAIR-83: 5'-GGAAAGCGAAGAAAUGCCCdTdT-3'
3'-dTdTCCUUUCGCUUCUUUACGGG-5' ;

LZ-PAIR-84: 5'-CCAAUGUUGUGACUUGGCCdTdT-3'
3'-dTdTGGUUACAACACUGAACCGG-5' ;

LZ-PAIR-85: 5'-GGCUAUCGCCGGUGCCGCGdTdT-3'
3'-dTdTCCGAUAGCGGCCACGGCGC-5' ;

LZ-PAIR-86: 5'-CCAGGACUGCACGAUGCUCdTdT-3'
3'-dTdTGGUCCUGACGUGCUACGAG-5' ;

LZ-PAIR-87: 5'-GCUAUGACUAGAUACUCUGdTdT-3'
3'-dTdTCGAUACUGAUCUAUGAGAC-5' ;

LZ-PAIR-88: 5'-GAUGCAUCUGGCAAAAGGGdTdT-3'
3'-dTdTCUACGUAGACCGUUUUCCC-5' ;

LZ-PAIR-89: 5'-CUGGCUAGGCAACAUCAUCdTdT-3'
3'-dTdTGACCGAUCCGUUGUAGUAG-5' ;

LZ-PAIR-90: 5'-CCUAGAUUGUCAGAUCUACdTdT-3'
3'-dTdTGGAUCUAACAGUCUAGAUG-5' ;

LZ-PAIR-91: 5'-CAUAGUUACUCUCCAGGUGdTdT-3'
3'-dTdTGUAUCAAUGAGAGGUCCAC-5' ;

LZ-PAIR-92: 5'-CGCGCUAGGCUACUGUCCCdTdT-3'
3'-dTdTGCGCGAUCCGAUGACAGGG-5' ;

LZ-PAIR-93: 5'-CCGGCUGCGUCCCAGUUGGdTdT-3'
3'-dTdTGGCCGACGCAGGGUCAACC-5' ;

LZ-PAIR-94: 5'-GGUGCCUACUCCUACUUUCdTdT-3'
3'-dTdTCCACGGAUGAGGAUGAAAG-5' ;

LZ-PAIR-95: 5'-CCCUAGUCACGGCUAGCUGdTdT-3'
3'-dTdTGGGAUCAGUGCCGAUCGAC-5' ;

LZ-PAIR-96: 5'-GCUGAUACUGGCCUCUCUGdTdT-3'
3'-dTdTCGACUAUGACCGGAGAGAC-5' ;

LZ-PAIR-97: 5'-CCGCCCCUCUCCCUCCCCCdTdT-3'
3'-dTdTGGCGGGGAGAGGGAGGGGG-5' ;

LZ-PAIR-98: 5'-CCUAGGGGUCUUUCCCCUCdTdT-3'
3'-dTdTGGAUCCCCAGAAAGGGGAG-5' ;

LZ-PAIR-99: 5'-CCCCCCACCUGGCGACAGGdTdT-3'
3'-dTdTGGGGGGUGGACCGCUGUCC-5' ;

LZ-PAIR-100: 5'-GGAUCUGAUCUGGGGCCUCdTdT-3'
3'-dTdTCCUAGACUAGACCCCGGAG-5' ;

LZ-PAIR-101: 5'-GGCUGGCAAGCGCCCCCCGdTdT-3'
3'-dTdTCCGACCGUUCGCGGGGGGC-5' ;

LZ-PAIR-102: 5'-CUCUGCCCCUCGGGGCACGdTdT-3'
3'-dTdTGAGACGGGGAGCCCCGUGC-5' ;

LZ-PAIR-103: 5'-CGCCCCUACUGGUAGCGGCdTdT-3'
3'-dTdTGCGGGGAUGACCAUCGCCG-5' ;

LZ-PAIR-104: 5'-CCCCAUCACGUACUCCACCdTdT-3'
3'-dTdTGGGGUAGUGCAUGAGGUGG-5' ;

LZ-PAIR-105: 5'-GCUACGCCUCCGGGAUCGGdTdT-3'
3'-dTdTCGAUGCGGAGGCCCUAGCC-5' ;

LZ-PAIR-106: 5'-GGCCUCGGACUCAAUGCUGdTdT-3'
3'-dTdTCCGGAGCCUGAGUUACGAC-5' ;

LZ-PAIR-107: 5'-GACCUUCACCAUUGAGACGdTdT-3'
3'-dTdTCUGGAAGUGGUAACUCUGC-5' ;

LZ-PAIR-108: 5'-CGAGCUCACGCCCGCCGAGdTdT-3'
3'-dTdTGCUCGAGUGCGGGCGGCUC-5' ;

LZ-PAIR-109: 5'-CCAGGCUACGGUGUGCGCCdTdT-3'
3'-dTdTGGUCCGAUGCCACACGCGG-5' ;

LZ-PAIR-110: 5'-CAUGUCGGCUGACCUGGAGdTdT-3'
3'-dTdTGUACAGCCGACUGGACCUC-5' ;

LZ-PAIR-111: 5'-GAAGAGUGCGCCUCACACCdTdT-3'
3'-dTdTCUUCUCACGCGGAGUGUGG-5' ;

LZ-PAIR-112: 5'-GCGGGAUACAAUAUUUAGCdTdT-3'
3'-dTdTCGCCCUAUGUUAUAAAUCG-5' ;

LZ-PAIR-113: 5'-GUAGGCGCCGGCAUCGCUGdTdT-3'
3'-dTdTCAUCCGCGGCCGUAGCGAC-5' ;

LZ-PAIR-114: 5'-GGCGCCCUAGUCGUCGGGGdTdT-3'
3'-dTdTCCGCGGGAUCAGCAGCCCC-5' ;

LZ-PAIR-115: 5'-CUUACCAUCACUCAGCUGCdTdT-3'
3'-dTdTGAAUGGUAGUGAGUCGACG-5' ;

LZ-PAIR-116: 5'-GUCAACGUGGGUACAAGGGdTdT-3'
3'-dTdTCAGUUGCACCCAUGUUCCC-5' ;

LZ-PAIR-117: 5'-GCACGCCCUCCCCGGCGCCdTdT-3'
3'-dTdTCGUGCGGGAGGGGCCGCGG-5' ;

LZ-PAIR-118: 5'-GGUUGCACAGGUACGCUCCdTdT-3'
3'-dTdTCCAACGUGUCCAUGCGAGG-5' ;

LZ-PAIR-119: 5'-GGCUGGCCAGGGGAUCUCCdTdT-3'
3'-dTdTCCGACCGGUCCCCUAGAGG-5' ;

LZ-PAIR-120: 5'-GACUCUUUCGAGCCGCUCCdTdT-3'
3'-dTdTCUGAGAAAGCUCGGCGAGG-5' ;

LZ-PAIR-121: 5'-GGGUGUCCAUUGCCGCCUGdTdT-3'
3'-dTdTCCCACAGGUAACGGCGGA-5' ;

LZ-PAIR-122: 5'-GCCCUCCGACGACGGCGACdTdT-3'
3'-dTdTCGGGAGGCUGCUGCCGCUG-5' ;

LZ-PAIR-123: 5'-CAGGCGCCCUGAUCACGCCdTdT-3'
3'-dTdTGUCCGCGGGACUAGUGCGG-5' ;

LZ-PAIR-124: 5'-GGAGAUGAAGGCGAAGGCGdTdT-3'
3'-dTdTCCUCUACUUCCGCUUCCGC-5' ;

LZ-PAIR-125: 5'-GACACCAAUUGACACCACCdTdT-3'
3'-dTdTCUGUGGUUAACUGUGGUGG-5' ;

LZ-PAIR-126: 5'-GAUUCCAAUACUCUCCUGGdTdT-3'
3'-dTdTCUAAGGUUAUGAGAGGACC-5' ;

LZ-PAIR-127: 5'-GCCAUAAGGUCGCUCACAGdTdT-3'
3'-dTdTCGGUAUUCCAGCGAGUGUC-5' ;

LZ-PAIR-128: 5'-GAUGCUCGUAUGCGGAGACdTdT-3'
3'-dTdTCUACGAGCAUACGCCUCUG-5' ;

LZ-PAIR-129: 5'-CGCGCACGAUGCAUCUGGCdTdT-3'
3'-dTdTGCGCGUGCUACGUAGACCG-5' ;

LZ-PAIR-130: 5'-CUCCAUCCUUCUAGCUCAGdTdT-3'
3'-dTdTGAGGUAGGAAGAUCGAGUC-5' ;

LZ-PAIR-131: 5'-GGAAACUUGGGGUACCGCCdTdT-3'
3'-dTdTCCUUUGAACCCCAUGGCGG-5' ;

LZ-PAIR-132: 5'-CCCGCUGGUUCAUGUGGUGdTdT-3'
3'-dTdTGGGCGACCAAGUACACCAC-5' ;

LZ-PAIR-133: 5'-AGUGGAUGAACCGGCUGAUdTdT-3'
3'-dTdTUCACCUACUUGGCCGACUA-5' ;

LZ-PAIR-134: 5'-GAUGAACCGGCUGAUAGCGdTdT-3'
3'-dTdTCUACUUGGCCGACUAUCGC-5' ;

LZ-PAIR-135: 5'-UCACGGAGGCUAUGACUAGdTdT-3'
3'-dTdTAGUGCCUCCGAUACUGAUC-5' ;

LZ-PAIR-136: 5'-GGGAGACAUAUAUCACAGCdTdT-3'
3'-dTdTCCCUCUGUAUAUAGUGUCG-5' ;

LZ-PAIR-137: 5'-AGACCCUUGCCGGCCCAAAdTdT-3'
3'-dTdTUCUGGGAACGGCCGGGUUU-5' ;

LZ-PAIR-138: 5'-CCAAGCUGCCCAUCAAUGCdTdT-3'
3'-dTdTGGUUCGACGGGUAGUUACG-5' ;

LZ-PAIR-139: 5'-CUCUUUGCUCCGUCACCACdTdT-3'
3'-dTdTGAGAAACGAGGCAGUGGUG-5' ;

LZ-PAIR-140: 5'-ACUUCUAUCCGUGGAGGAAdTdT-3'
3'-dTdTUGAAGAUAGGCACCUCCUU-5' ;

LZ-PAIR-141: 5'-UUGACACCACCAUCAUGGCdTdT-3'
3'-dTdTAACUGUGGUGGUAGUACCG-5' ;

LZ-PAIR-142: 5'-UGCCUGGAAAGCGAAGAAAdTdT-3'
3'-dTdTACGGACCUUUCGCUUCUUU-5' ;

LZ-PAIR-143: 5'-UGACAUCCGUGUUGAGGAGdTdT-3'
3'-dTdTACUGUAGGCACAACUCCUC-5' ;

LZ-PAIR-144: 5'-UGAGCACACUUCCUAAACCdTdT-3'
3'-dTdTACUCGUGUGAAGGAUUUGG-5' ;

LZ-PAIR-145: 5'-GUUUACUUGUUGCCGCGCAdTdT-3'
3'-dTdTCAAAUGAACAACGGCGCGU-5' ;

LZ-PAIR-146: 5'-CCGACCUCAUGGGGUACAUdTdT-3'
3'-dTdTGGCUGGAGUACCCCAUGUA-5' ;

LZ-PAIR-147: 5'-CUCUUCAACUGGGCGGUGAdTdT-3'
3'-dTdTGAGAAGUUGACCCGCCACU-5' ;

LZ-PAIR-148: 5'-ACAGGACGUCAAGUUCCCGdTdT-3'
3'-dTdTUGUCCUGCAGUUCAAGGGC-5' ;

LZ-PAIR-149: 5'-GGGUAAGGUCAUCGAUACCdTdT-3'
3'-dTdTCCCAUUCCAGUAGCUAUGG-5' ;

LZ-PAIR-150: 5'-CUCCACCAAAACAUCGUGGdTdT-3'
3'-dTdTGAGGUGGUUUUGUAGCACC-5' .

Like the other dsRNAs listed above, the 5'-3' nucleotide sequences in the LZ dsRNA oligonucleotide pairs shown above correspond to, i.e., are homologous to or have sequence identity with, corresponding contiguous 5'-3' target polynucleotide sequences within the positive strand RNA genome of HCV (genotype 1b). Ten of these LZ pairs are also present among the dsRNA oligonucleotides having a G+C% between 30% and 70%, presented above, i.e., LZ pairs 6, 7, 124, 133, 134, 135, 136, 146, 148, and 149. The LZ pair dsRNAs for silencing of HCV gene expression and inhibiting HCV infection, replication, and/or pathogenesis have nucleotide sequences corresponding to contiguous nucleotide sequences spaced approximately every 100 nucleotides in the HCV genome,

including the coding regions for all the structural and nonstructural proteins, as well as the non-coding regions such as 3'-untranslated region (3'-UTR), internal ribosome entry site (IRES), and the 5'-untranslated region (5'-UTR).

Specifically, dsRNA oligonucleotide LZ pairs 1 to 4 and 97 to 100 have sequences corresponding to the 5'-UTR region; dsRNA oligonucleotide LZ pairs 5 to 9, 144 to146, and 148 to 149 have sequences corresponding to the coat (capsid) protein coding region; dsRNA oligonucleotide LZ pairs 10 to 15 have sequences corresponding to the E1 protein coding region; dsRNA oligonucleotide LZ pairs 16 to 26 and 150 have sequences corresponding to the E2 protein coding region; dsRNA oligonucleotide LZ pair 27 has a sequence corresponding to the p7 protein coding region; dsRNA oligonucleotide pairs LZ 28 to 34 have sequences corresponding to the NS2 (nonstructural) protein coding region; dsRNA oligonucleotide LZ pairs 35 to 53, 101 to 110, and 137 have sequences corresponding to the NS3 protein coding region; dsRNA oligonucleotide LZ pairs 54 to 55 have sequences corresponding to the NS4A protein coding region; dsRNA oligonucleotide LZ pairs 56 to 63, 111 to 115, and 133 to 134 have sequences corresponding to the NS4B protein coding region; dsRNA oligonucleotide LZ pairs 64 to 76 and 116 to 122 have sequences corresponding to the NS5A protein coding region; dsRNA oligonucleotide LZ pairs 77 to 94, 123 to 132, 135 to 143, and 147 have sequences corresponding to the NS5B protein coding region; and dsRNA oligonucleotide LZ pairs 95 to 96 have sequences corresponding to the 3'-UTR region.

As shown in Example 1, below, LZ-133 and LZ-135 each exhibit good anti-HCV inhibitory activity *in vitro*. When used in combination, these two dsRNAs would be effective inhibitors of a subset of 101 of the 147 sequenced HCV isolates listed and analyzed above. Most of these 101 sequenced isolates are likely to be HCV genotype 1a or 1b, which are the prevalent genotypes in the United States, Europe, and Japan. In order to treat infections caused by the other 46 members of the set of 147 sequenced HCV isolates, one can further employ different sets of multiple dsRNA oligonucleotides that target HCV RNA polynucleotide sequences present in the 46 sequenced isolates lacking sequences homologous to LZ-133 and LZ-135. When used in combination with LZ-133 and LZ-135, the multiple dsRNAs of each additional set will permit targeting of RNA polynucleotide sequences in all 147 sequenced isolates of HCV used in this analysis. If necessary, different sets of dsRNAs can be used together as well.

Ten non-limiting, exemplary sets of such dsRNA oligonucleotides are shown below. The indicated positions are obtained by aligning the 147 sequenced HCV isolates as described above. Some dsRNAs listed below may correspond to HCV sequences present at different positions in different genomes, although the chance of this is low. Other useful sets can be readily designed following the same strategy. The use of sequence information in this manner makes possible the design of multiple dsRNAs for use in preventing or treating HCV infections caused by HCV of many different genotypes.

dsRNA Set 1

position 100-118
5'-GUUUACUUGUUGCCGCGCA-3'
3'-CAAAUGAACAACGGCGCGU-5'

position 392-410
5'-CCGACCUCAUGGGGUACAU-3'
3'-GGCUGGAGUACCCCAUGUA-5'

position 6428-6446
5'-AGAUACACCGAUUUGCCCC-3'
3'-UCUAUGUGGCUAAACGGGG-5'

position 8426-8444
5'-UCACGGAGGCUAUGACCAG-3'
3'-AGUGCCUCCGAUACUGGUC-5'

dsRNA Set 2

position 9010-9028
5'-CUCAAACUCACUCCAUUGC-3'
3'-GAGUUUGAGUGAGGUAACG-5'

position 9086-9104
5'-ACAUUUAUCACAGCGUGUC-3'
3'-UGUAAAUAGUGUCGCACAG-5'

position 9127-9145
5'-CUCCUGUGCCUACUCCUAC-3'
3'-GAGGACACGGAUGAGGAUG-5'

dsRNA Set 3

position 102-120
5'-UUACUUGUUGCCGCGCAGG-3'
3'-AAUGAACAACGGCGCGUCC-5'

position 1718-1736
5'-GUGCUCCACCAUGCCACAU-3'
3'-CACGAGGUGGUACGGUGUA-5'

position 9008-9026
5'-AGCUCAAACUCACUCCAUU-3'
3'-UCGAGUUUGAGUGAGGUAA-5'

position 9085-9103
5'-GACAUUUAUCACAGCGUGU-3'
3'-CUGUAAAUAGUGUCGCACA-5'

dsRNA Set 4

position 55-73
5'-CCACAGGACGUCAAGUUCC-3'
3'-GGUGUCCUGCAGUUCAAGG-5'

position 356-374
5'-UGGGUAAGGUCAUCGAUAC-3'
3'-ACCCAUUCCAGUAGCUAUG-5'

position 4213-4231
5'-CUGAUUUUCUGCCACUCAA-3'
3'-GACUAAAAGACGGUGAGUU-5'

position 8425-8443
5'-UUCACGGAGGCUAUGACCA-3'
3'-AAGUGCCUCCGAUACUGGU-5'

dsRNA Set 5

position 54-72
5'-CCCACAGGACGUCAAGUUC-3'
3'-GGGUGUCCUGCAGUUCAAG-5'

position 358-376
5'-GGUAAGGUCAUCGAUACCC-3'
3'-CCAUUCCAGUAGCUAUGGG-5'

position 4622-4640
5'-GGUACGAUCUCACACCAGC-3'
3'-CCAUGCUAGAGUGUGGUCG-5'

position 8429-8447
5'-CGGAGGCUAUGACCAGGUA-3'
3'-GCCUCCGAUACUGGUCCAU-5'

dsRNA Set 6

position 357-375
5'-GGGUAAGGUCAUCGAUACC-3'
3'-CCCAUUCCAGUAGCUAUGG-5'

position 395-413
5'-ACCUCAUGGGGUACAUCCC-3'
3'-UGGAGUACCCCAUGUAGGG-5'

position 634-652
5'-AUCACCUGGCAGCUCACUA-3'
3'-UAGUGGACCGUCGAGUGAU-5'

position 8424-8442
5'-CUUCACGGAGGCUAUGACC-3'
3'-GAAGUGCCUCCGAUACUGG-5'

dsRNA Set 7

position 56-74
5'-CACAGGACGUCAAGUUCCC-3'
3'-GUGUCCUGCAGUUCAAGGG-5'

position 359-377
5'-GUAAGGUCAUCGAUACCCU-3'
3'-CAUUCCAGUAGCUAUGGGA-5'

position 3262-3280
5'-AUCUCGGGGGUUUUGUGGA-3'
3'-UAGAGCCCCCAAAACACCU-5'

position 8428-8446
5'-ACGGAGGCUAUGACCAGGU-3'
3'-UGCCUCCGAUACUGGUCCA-5'

dsRNA Set 8

position 355-373
5'-UUGGGUAAGGUCAUCGAUA-3'
3'-AACCCAUUCCAGUAGCUAU-5'

position 8423-8441
5'-CCUUCACGGAGGCUAUGAC-3'
3'-GGAAGUGCCUCCGAUACUG-5'

position 9007-9025
5'-AAGCUCAAACUCACUCCAU-3'
3'-UUCGAGUUUGAGUGAGGUA-5'

position 9151-9169
5'-GUAGGGGUAGGCAUCUUUU-3'
3'-CAUCCCCAUCCGUAGAAAA-5'

dsRNA Set 9

position 508-526
5'-CCUGGUUGCUCUUUCUCUA-3'
3'-GGACCAACGAGAAAGAGAU-5'

position 3031-3049
5'-UUGUGCGGGUUGCCCGUUU-3'
3'-AACACGCCCAACGGGCAAA-5'

position 8421-8439
5'-AGCCUUCACGGAGGCUAUG-3'
3'-UCGGAAGUGCCUCCGAUAC-5'

position 9009-9027
5'-GCUCAAACUCACUCCAUUG-3'
3'-CGAGUUUGAGUGAGGUAAC-5'

position 9152-9170
5'-UAGGGGUAGGCAUCUUUUU-3'
3'-AUCCCCAUCCGUAGAAAAA-5'

dsRNA Set 10

position 57-75
5'-ACAGGACGUCAAGUUCCCG-3'
3'-UGUCCUGCAGUUCAAGGGC-5'

position 354-372
5'-UUUGGGUAAGGUCAUCGAU-3'
3'-AAACCCAUUCCAGUAGCUA-5'

position 389-407
5'-UUGCCGACCUCAUGGGGUA-3'
3'-AACGGCUGGAGUACCCCAU-5'

position 8416-8434
5'-CUGCGAGCUUUCACGGAAG-3'
3'-GACGCUCGAAAGUGCCUUC-5'

position 8422-8440
5'-GCCUUCACGGAGGCUAUGA-3'
3'-CGGAAGUGCCUCCGAUACU-5'

Assay systems that can be used to determine *in vitro* IC_{50} values for the HCV dsRNAs disclosed herein, and therefore to identify useful anti-HCV therapeutics, are described in, or can be developed using the information disclosed in, Lohmann et al., *Science* 285:110-113 (1999); Blight et al., *Science* 290: 1972-1974 (2000); Guo et al., *J. Virol.* 75:8516-8523 (2001); Applicants' copending application PCT/US01/26008, filed August 31, 2001; and as described below in Examples 1 and 2.

One or more of the dsRNAs disclosed herein can be administered prophylactically to a patient at risk for contracting an HCV infection, or to a patient suffering from an HCV infection, in order to silence expression of the corresponding HCV gene(s) or otherwise inhibit HCV infection, replication, and/or pathogenesis, thereby preventing or treating the infection, respectively. It should be noted that the dsRNAs disclosed herein can be used alone, or in various combinations to target one or more HCV target RNA polynucleotide sequences.

For HCV prevention or treatment, one can employ dsRNAs that target one region of one HCV target RNA polynucleotide sequence; multiple dsRNAs that target more than one region of one HCV target RNA polynucleotide sequence; multiple dsRNAs that target single regions of multiple HCV target RNA polynucleotide sequences; multiple dsRNAs that target multiple regions of multiple HCV target RNA polynucleotide sequences; and combinations of these techniques.

As noted below in the section entitled "Formulation and Administration of dsRNAs," a number of different means can be employed to administer the dsRNA

oligonucleotide pair(s) to a patient. For example, one method involves genetically modifying hepatocytes in culture with retroviral vectors and implanting them back into the patient's liver (Grossman et al., *Nature Genet.* 6: 335-341(1994)), or delivering them directly to the liver whereby a partial hepatectomy induces hepatocyte division (Kay et al., *Hum. Gene Ther.* 3:641-647(1992)). Transient high levels of foreign gene expression are attainable by injection of adenoviral vectors into the portal or systemic circulatory systems (Stratford-Perricaudet et al., *Hum. Gene Ther.* 1:241-256 (1990)). Naked DNA injection in isotonic solutions into the liver parenchyma after treatment with dexamethasone has been used to achieve transient foreign gene expression (Hickman et al., *Hum. Gene Ther.* 5: 1477-1483(1994)). Liposomes containing plasmid DNA can be delivered by intraportal routes for expression in the liver (Kaneda et al., *Science* 243: 375-378(1989)). Gene targeting using synthetic glycopeptide-based delivery of plasmids that targets the asialoglycoprotein receptor on hepatocytes, then utilizes an endosomolytic peptide to release the plasmids from the endosomes after they are taken up by the hepatocyte receptors, has also been described (Anwer et al., *Pharmaceutical Res.* 17(4):451-459). Intranasal administration may also be particularly effective.

The amount (dose) of any one or more of the foregoing dsRNAs effective in reducing HCV gene expression or otherwise preventing or treating HCV infection in a patient, as well as the most effective formulation, route of administration, and treatment regimen, can be determined by monitoring HCV titers in patients undergoing treatment. This can be performed, for example, by monitoring HCV RNA in patients' serum by slot-blot, dot-blot, or RT-PCR techniques such as the Amplicor® HCV Test, version 2.0, or the Cobas Amplicor™ HCV Test, version 2.0 (Roche Diagnostics Corp., Indianapolis, IN), or by measurement of HCV surface or other antigens. These methods can be used in combination with the monitoring of levels of various diagnostically useful liver enzymes, for example alanine aminotransferase (ALT) or aspartate aminotransferase (AST), in the blood.

Formulation and Administration of dsRNAs

dsRNAs of the present invention can be formulated as compositions comprising the active compounds and a buffer, carrier, diluent, or excipient, which can be sterile and

non-toxic to human cells. For therapeutic purposes, the buffer, etc. should be pharmaceutically acceptable.

dsRNAs can be introduced into cells in a number of different ways. For example, the dsRNA can be administered by microinjection; bombardment by microparticles covered by the dsRNA; soaking the cells in a solution of the dsRNA; electroporation of cells in the presence of the dsRNA; liposome-mediated delivery of dsRNA; transfection mediated by chemicals such as calcium phosphate, Oligofectamine™, etc.; viral infection; transformation; and the like. The dsRNA can be introduced along with components that enhance RNA uptake by the cells, stabilize the annealed strands, or otherwise increase the inhibition of function of the target polynucleotide sequence. In the case of a cell culture or tissue explant, the cells are conveniently incubated in a solution containing the dsRNA, or subjected to lipid-mediated transfection.

In the case of a human or animal patient undergoing prophylaxis or treatment for an HCV infection, the dsRNA can be conveniently introduced by injection or perfusion into a cavity or interstitial space of the patient, or systemically via oral (including buccal or sublingual), topical (including buccal, sublingual, or transdermal), parenteral (including subcutaneous, intramuscular, intravenous, or intradermal administration), intra-pulmonary, vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration. In addition, the dsRNA can be administered via an implantable extended release device. Methods for oral introduction include direct mixing of dsRNA with food of the patient, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the patient to be treated. Different routes of administration can be combined, if necessary or desired, and if multiple dsRNAs are employed, they can be administered concurrently or sequentially.

Alternatively, dsRNA can be supplied to cells within the patient indirectly by introducing one or more vectors that encode both single strands of a dsRNA (or, in the case of a self-complementary RNA, the single self-complementary strand) into the cells. Preferably, the vector contains 5' and 3' regulatory elements that facilitate transcription of the coding sequence. Single stranded RNA is transcribed inside the cell, and dsRNA forms spontaneously and attenuates expression or function of the target pathogen gene or other target polynucleotide sequence. Methods for supplying a cell with dsRNA by

introducing a vector from which it can be transcribed are disclosed in WO 99/32619, and reviewed in Tuschl, *Nature Biotechnology*, 20:446-448 (2002).

Viral vector-mediated gene transfer has been used successfully in mouse models and human clinical trials. See Fujiwara et al., *Cancer Research*, 54:2287-2291 (1994), and Roth et al., *Nature Medicine*, 2:985-991 (1996). Mountain, *TIBTECH*, 18:119-128 (2000) discusses recent examples of gene therapy with clinical benefit progressing to Phase II clinical studies using cationic lipids, adenovirus, retrovirus, and adeno-associated virus vectors, as well as naked DNA. Recent U.S. Patents claiming methods of gene therapy include Nos. 6,080,728 and 6,087,164. PCT International Publication WO 00/63364 describes a variety of viral delivery agents.

Recent books and reviews describing methods of delivering nucleic acid therapeutics include A. Rolland, *Advanced Gene Delivery: From Concept to Pharmaceutical Products* (1999); G. Gregoriadis, *Targeting of Drugs 5: Strategies for Oligonucletide and Gene Delivery in Therapy* (1997); Hughes et al., *Drug Discovery Today* 6(6):303-315 (2001); Lambert et al., *Adv. Drug Deliv. Rev.* 47:99-112 (2001); Wraight et al., *Pharmacol. & Therapeutics* 90:89-104 (2001); Akhtar et al., *Adv. Drug Deliv. Rev.* 44:3-21 (2000); and Sioud, *Int. J. Mol. Med.* 3:381-384 (1999). PCT International Publication Nos. WO 01/29058, WO 01/04313, WO 01/16312, WO 99/05094, WO 99/04819 also describe methods of delivering nucleic acid therapeutics. European Patent Applications EP 1 086 116 A1 and EP 1 080 225 A1 disclose compositions and methods for the pulmonary delivery of nucleic acids; EP 1 080 103 A1 discloses compositions and methods for non-parenteral delivery of oligonucleotides.

McCaffrey et al. (*Nature* 418:38-39 (2002)) recently described suppression of transgene expression, including targeting of an HCV NS5B fusion protein, in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed *in vivo* from DNA templates. The authors used a modification of hydrodynamic transfection methods to deliver naked siRNAs to the livers of adult mice.

Dosing and Treatment Regimen

Determination of the optimal amounts of dsRNAs to be administered to human or animal patients in need of prevention or treatment of HCV infections, as well as methods of administering therapeutic or pharmaceutical compositions comprising such dsRNA

oligonucleotides, is within the skill of those in the pharmaceutical art. Dosing of a human or animal patient is dependent on the nature of the HCV genotype; the nature of the infected cell or tissue; the patient's condition; body weight; general health; sex; diet; time, duration, and route of administration; rates of absorption, distribution, metabolism, and excretion of the dsRNA; combination with other drugs; severity of the infection; and the responsiveness of the disease state being treated. The amount of dsRNA administered also depends on the nature of the HCV target polynucleotide sequence and the nature of the dsRNA, and can readily be optimized to obtain the desired level of effectiveness. The course of treatment can last from several days to several weeks or several months, or until a cure is effected or an acceptable diminution or prevention of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient in conjunction with the effectiveness of the treatment. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies, and repetition rates. Optimum dosages can vary depending on the relative potency of individual dsRNA compounds, and can generally be estimated based on IC_{50} values found to be effective in *in vitro* and *in vivo* animal models. Effective amounts of dsRNAs for the treatment or prevention of HCV infections, delivery vehicles containing dsRNAs or constructs encoding the same, agonists, and treatment protocols, can be determined by conventional means. For example, the medical or veterinary practitioner can commence treatment with a low dose of one or more dsRNAs in a subject or patient in need thereof, and then increase the dosage, or systematically vary the dosage regimen, monitor the effects thereof on the patient or subject, and adjust the dosage or treatment regimen to maximize the desired therapeutic effect. Further discussion of optimization of dosage and treatment regimens can be found in Benet et al., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Ninth Edition, Hardman *et al.*, Eds., McGraw-Hill, New York, (1996), Chapter 1, pp. 3-27; Thummel et al., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Tenth Edition, Hardman *et al.*, Eds., McGraw-Hill, New York, (2001), Appendix II, pp. 1917-1923; and L.A. Bauer, in *Pharmacotherapy, A Pathophysiologic Approach*, Fourth Edition, DiPiro et al., Eds., Appleton & Lange, Stamford, Connecticut, (1999), Chapter 3, pp.21-43, and the references cited therein, to which the reader is referred.

In the context of the present invention, the terms "treatment," "therapeutic use," or "treatment regimen" as used herein are meant to encompass prophylactic, palliative, and therapeutic modalities of administration of one or more dsRNAs of the present invention, and include any and all uses of the presently claimed dsRNA compounds that remedy a disease state, condition, symptom, or disorder caused by HCV, or which prevent, hinder, retard, or reverse the progression of symptoms, conditions, or disorders associated with HCV infection. Thus, any prevention, amelioration, alleviation, reversal, or complete elimination of an undesirable disease state, symptom, condition, or disorder associated with HCV infection is encompassed by the present invention.

A particular treatment regimen may last for a period of time that will vary depending upon the nature of the particular disease or disorder, its severity, and the overall condition of the patient, and may involve administration of dsRNA-containing compositions from once to several times daily for several days, weeks, months, or longer. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms or conditions of the disorder or disease state. The dosage of the composition can either be increased in the event the patient does not respond significantly to current dosage levels, or the dose can be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

An optimal dosing schedule is used to deliver a therapeutically effective amount of the dsRNA oligonucleotides of the present invention. For the purposes of the present invention, the term "therapeutically effective amount" with respect to a dsRNA oligonucleotide disclosed herein refers to an amount of dsRNA oligonucleotide(s) that is effective to achieve an intended purpose, preferably without undesirable side effects such as toxicity, irritation, or allergic response. Although individual patient needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies (A.S. Katocs, *Remington: The Science and Practice of Pharmacy,* 19th Ed., A.R. Gennaro, ed., Mack Publishing Co., Easton, Pa., (1995), Chapter 30). Generally, the dosage required to provide a therapeutically effective amount of a pharmaceutical composition, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of

treatment, the nature of concurrent therapy (if any), and the nature and scope of the desired effect(s) (A. S. Nies, *Goodman & Gilman's The Pharmacological Basis of Therapeutics,* Tenth Edition, Hardman et al., eds., McGraw-Hill, New York, N.Y., 2001, Chapter 3).

Prophylactic modalities for high risk individuals are also encompassed by the present invention. As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., living or working environment or conditions, intravenous drug use, past history of blood transfusion, etc., that there is a significantly higher than normal probability of being susceptible to, or contracting, HCV infection, or the onset or recurrence of an HCV-related disease or disorder. As part of a treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent infection or the onset or recurrence of the disease, disorder, symptom, or condition. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition of the present invention that produces an effect observed as the prevention of HCV infection, or the onset or recurrence of a disease, disorder, symptom, or condition associated therewith. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have compared to the effect observed when a second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

For therapeutic use, one or more dsRNA oligonucleotides as disclosed herein is(are) administered to a patient suspected of suffering from an HCV infection in an amount effective to reduce the symptomology of the associated disease. One skilled in the art can determine optimum dosages and treatment schedules for such treatment regimens, as discussed above.

Whether used prophylactically, palliatively, or therapeutically, a dsRNA of the present invention for use in modulating an HCV target RNA polynucleotide sequence should designed to be homologous to a preselected region of that target RNA polynucleotide sequence.

Individual dsRNAs can be administered in an amount that allows delivery of at least one copy thereof per infected cell. It should be noted that multiple dsRNAs, directed at different regions of a single HCV target polynucleotide sequence, or at different HCV target polynucleotide sequences or polynucleotide regions involved in

viral infection, replication, and/or pathogenesis, can be employed in combination therapy with one another. For purposes of the present invention, dsRNAs as described herein can be introduced into an organism such as an infected human or animal in an amount effective to ameliorate, inhibit, reverse, or completely eliminate the adverse effects of HCV, i.e., "a therapeutically effective amount." A therapeutically effective amount can thus be an amount capable of delivering at least one copy of the dsRNA (or of each one of multiple dsRNAs if multiple dsRNA oligonucleotides are employed) per infected cell. Higher doses, e.g., about 5, about 10, about 25, about 50, about 100, about 250, about 500, about 1000, about 5,000, about 10,000, about 100,000, or about 1,000,000 copies or more of dsRNA material per infected cell can also be used. In general, a patient, whether human or animal, in need of prophylaxis or therapy is administered a composition, preferably a pharmaceutical composition, comprising one or more dsRNAs of the present invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from about 0.01 μg to about 100 g per kg of body weight, or from about 0.01 μg to about 100 mg per kg of body weight, depending on the age and condition of the patient, and the severity of the symptom, condition, disorder, or disease state being treated.

In the case of administration of dsRNAs to a cell culture or to cells in a tissue by methods other than injection, for example by soaking, electroporation, or lipid-mediated transfection, the cells are preferably exposed to similar levels of dsRNA in the medium. For example, 8-10 mL of cell culture or tissue can be contacted with about 20 x 10^6 to about 2000 x 10^6 molecules of dsRNA, more preferably about 10 x 10^6 to about 500 x 10^6 molecules of dsRNA, for effective attenuation of gene expression or attenuation of target RNA polynucleotide sequence function.

Combination Therapy for the Treatment of HCV Infections

The antiviral dsRNAs disclosed herein can be used alone, together in combinations with one another, or alone or together in further combination with other molecules that directly exhibit or indirectly elicit antiviral activity. The terms "antiviral" or "antiviral activity" refer to the capacity of a molecule, when present, to completely inhibit or reduce accumulation of viral virions compared to viral virion accumulation in the absence of such molecule, and/or the capacity of a molecule to reduce or ameliorate symptoms, conditions, or disorders associated with virus infection or pathogenesis in

patients. Molecules having antiviral activity, including dsRNAs of the present invention, encompass those that disrupt one or more steps in viral infection, replication, and/or pathogenesis, including those that evoke immunomodulating and antiproliferative actions in host cells. Molecules having antiviral activity can inhibit virus-specific replicative events such as, but not limited to, virus-directed nucleic acid or protein synthesis. Steps or stages of virus infection, replication, and/or pathogenesis at which molecules having antiviral activity can act include cell entry (e.g., attachment; penetration); uncoating and release of the viral genome; replication of the viral genome (e.g., replication of either strand of the viral DNA or RNA genome; transcription of viral messenger RNA); translation of virus proteins; post-translational modification of virus proteins (e.g., proteolytic cleavage; glycosylation); intracellular transport of viral proteins; assembly of virion components; and release of viral particles (e.g., budding). Classes of molecules having antiviral activity include, but are not limited to, soluble receptor decoys and antireceptor antibodies; ion channel blockers, capsid stabilizers, and fusion protein inhibitors; inhibitors of viral polymerases, reverse transcriptases, helicases, primases, or integrases; antisense oligonucleotides and ribozymes; immunomodulating and immunostimulating agents, including cytokines such as interferons, as well as peptide agonists, steroids, and classic drugs such as levamisole; inhibitors of regulatory proteins; protease inhibitors; assembly protein inhibitors; and antiviral antibodies and cytotoxic lymphocytes. Compounds having antiviral activity contemplated for use in the combination compositions and methods of combination therapy disclosed herein include, but are not limited to, immunomodulatory molecules, including immunostimulatory cytokines, and other compounds known to have antiviral activity, such as various antiviral nucleosides and nucleotides. The term "antivirus effective amount" or "pharmaceutically effective amount" as applied to such molecules refers to an amount of a compound, or combination of compounds as disclosed herein, effective in reducing or ameliorating symptoms, conditions, or disorders associated with HCV infection or associated pathogenesis in patients, or in reducing viral levels *in vitro* or *in vivo*.

Immunomodulatory molecules contemplated for use in combination with the antiviral dsRNAs disclosed herein include, but are not limited to, interferon-alpha 2B (Intron A, Schering Plough); Rebatron (Schering Plough, Interferon-alpha 2B + Ribavirin); pegylated interferon alpha (Reddy et al., *Hepatology* 33:433-438 (2001));

consensus interferon (Kao et al., *Gastroenterol. Hepatol.* 15:1418-1423 (2000)); interferon-alpha 2A (Roferon A; Roche); lymphoblastoid or "natural" interferon; interferon gamma; interferon tau (Clayette et al., *Pathol. Biol. (Paris)* 47:553-559 (1999)); platelet derived growth factor; colony stimulating factors such as G-CSF and GM-CSF; tumor necrosis factor (TNF); epidermal growth factor (EGF); and interleukins such as interleukin 1, interleukin 2 (Davis et al., *Seminars in Liver Disease* 19:103-112 (1999)); interleukin 4; interleukin 6 (Davis et al., *Seminars in Liver Disease* 19:103-112 (1999)); interleukin 8; interleukin 10; and interleukin 12 (Davis et al., *Seminars in Liver Disease* 19:103-112 (1999)); Ribavirin; and compounds that enhance the development of a type 1 helper T cell response (Davis et al., *Seminars in Liver Disease* 19:103-112 (1999)). Interferons may ameliorate viral infections by exerting direct antiviral effects and/or by modifying the immune response to infection. The antiviral effects of interferons are often mediated through inhibition of viral penetration or uncoating, synthesis of viral RNA, translation of viral proteins, and/or viral assembly and release. Other potentially useful agents include fibroblast growth factor; surface active agents such as immune-stimulating complexes (ISCOMS); Freund's incomplete adjuvant; LPS analogs, including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicular complexes such as squalene; and hyaluronic acid.

Compounds that stimulate the synthesis of interferon in cells (Tazulakhova et al., *J. Interferon Cytokine Res.* 21:65-73)) include, but are not limited to, long double stranded RNA, alone or complexed with tobramycin, and Imiquimod (3M Pharmaceuticals) (Sauder, *J. Am. Acad. Dermatol.* 43:S6-11 (2000)).

Other compounds known to have, or that may have, antiviral activity by virtue of non-immunomodulatory mechanisms include, but are not limited to, Ribavirin (ICN Pharmaceuticals); inosine 5'-monophosphate dehydrogenase inhibitors (VX-497, being developed by Vertex Pharmaceuticals); amantadine and rimantadine (Younossi et al., *Seminars in Liver Disease* 19:95-102 (1999)); LY217896 (U.S. Patent 4,835,168; (Colacino et al., *Antimicrobial Agents & Chemotherapy* 34:2156-2163 (1990)); and LY311912, LY314177, and LY334177 (U.S. Patent 6,127,422).

Compounds useful in treating HCV infections in combination with the anti-HCV dsRNAs disclosed herein include the HCV NS3 serine protease inhibitors disclosed in Applicant's copending application PCT/US01/26008, filed August 31, 2001; in PCT

International Publication Nos. WO 00/09558, WO 00/09543, WO 99/64442, WO 99/07733, WO 99/07734, WO 99/50230, WO98/46630, WO 98/17679, and WO 97/43310; in United States Patent No. 5,990,276; and in Llinás-Brunet et al., *Bioorg. Med. Chem. Lett.*, 8:1713-1718 (1998); Han et al., *Bioorg. Med. Chem. Lett.*, 10:711-713 (2000); Dunsdon et al., *Bioorg. Med. Chem. Lett.*, 10:1571-1579 (2000); Llinás-Brunet et al., *Bioorg. Med. Chem. Lett.*, 10:2267-2270 (2000); and LaPlante et al., *Bioorg. Med. Chem. Lett.*, 10:2271-2274 (2000). Gemcitabine (2',2'-Difluorodeoxycytidine) is another compound potentially useful as an HCV antiviral.

Formulations, doses, and routes of administration for the foregoing molecules are either taught in the cited references, or are well-known in the art as disclosed, for example, in F.G. Hayden, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Tenth Edition, Hardman et al., Eds., McGraw-Hill, New York (2001), Chapter 50, pp. 1313-1347, and Krensky et al., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Tenth Edition, Hardman et al., Eds., McGraw-Hill, New York (2001), Chapter 53, pp. 1463-1484, and the references cited therein. Alternatively, once a compound that exhibits antiviral activity has been identified, a pharmaceutically effective amount of that compound can be determined using techniques that are well known to the skilled artisan. Note, for example, Benet et al., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Ninth Edition, Hardman et al., Eds., McGraw-Hill, New York (1996), Chapter 1, pp. 3-27, and the references cited therein. Thus, the appropriate formulations, dose(s) range, and dosing regimens of such a compound can be easily determined by routine methods.

The drugs of the present invention, either alone or in combination, can be provided to a cell or cells, or to a human or animal patient, either in separate pharmaceutically acceptable formulations administered simultaneously or sequentially, formulations containing more than one therapeutic agent, or by an assortment of single agent and multiple agent formulations. However administered, these drug combinations form an anti-HCV effective amount of components.

A large number of other immunomodulators and immunostimulants that can be used in the methods of the present invention are currently available. These include: AA-2G; adamantylamide dipeptide; adenosine deaminase, Enzon; adjuvant, Alliance; adjuvants, Ribi; adjuvants, Vaxcel; Adjuvax; agelasphin-11; AIDS therapy, Chiron; algal

glucan, SRI; algammulin, Anutech; Anginlyc; anticellular factors, Yeda; Anticort; antigastrin-17 immunogen, Ap; antigen delivery system, Vac; antigen formulation, IDBC; antiGnRH immunogen, Aphton; Antiherpin; Arbidol; azarole; Bay-q-8939; Bay-r-1005; BCH-1393; Betafectin; Biostim; BL-001; BL-009; Broncostat; Cantastim; CDRI-84-246; cefodizime; chemokine inhibitors, ICOS; CMV peptides, City of Hope; CN-5888; cytokine-releasing agent, St; DHEAS, Paradigm; DISC TA-HSV; J07B; I01A; I01Z; ditiocarb sodium; ECA-10-142; ELS-1; endotoxin, Novartis; FCE-20696; FCE-24089; FCE-24578; FLT-3 ligand, Immunex; FR-900483; FR-900494; FR-901235; FTS-Zn; G-proteins, Cadus; gludapcin; glutaurine; glycophosphopeptical; GM-2; GM-53; GMDP; growth factor vaccine, EntreM; H-BIG, NABI; H-CIG, NABI; HAB-439; Helicobacter pylori vaccine; herpes-specific immune factor; HIV therapy, United Biomed; HyperGAM+CF; ImmuMax; Immun BCG; immune therapy, Connective; immunomodulator, Evans; immunomodulators, Novacell; imreg-1; imreg-2; Indomune; inosine pranobex; interferon, Dong-A (alpha2); interferon, Genentech (gamma); interferon, Novartis (alpha); interleukin-12, Genetics Ins; interleukin-15, Immunex; interleukin-16, Research Cor; ISCAR-1; J005X; L-644257; licomarasminic acid; LipoTher; LK-409; LK-410; LP-2307; LT (R1926); LW-50020; MAF, Shionogi; MDP derivatives, Merck; met-enkephalin, TNI; methylfurylbutyrolactones; MIMP; mirimostim; mixed bacterial vaccine, Tem; MM-1; moniliastat; MPLA, Ribi; MS-705; murabutide; murabutide, Vacsyn; muramyl dipeptide derivative; muramyl peptide derivatives; myelopid; 563; NACOS-6; NH-765; NISV, Proteus; NPT-16416; NT-002; PA-485; PEFA-814; peptides, Scios; peptidoglycan, Pliva; Perthon, Advanced Plant; PGM derivative, Pliva; Pharmaprojects No. 1099; No. 1426; No. 1549; No. 1585; No. 1607; No. 1710; No. 1779; No. 2002; No. 2060; No. 2795; No. 3088; No. 3111; No. 3345; No. 3467; No. 3668;No. 3998; No. 3999; No. 4089; No. 4188; No. 4451; No. 4500; No. 4689; No. 4833; No. 494; No. 5217; No. 530; pidotimod; pimelautide; pinafide; PMD-589; podophyllotoxin, Conpharm; POL-509; poly-ICLC; poly-ICLC, Yamasa Shoyu; PolyA-PolyU; Polysaccharide A; protein A, Berlox Bioscience; PS34WO; *Pseudomonas* MAbs, Teijin; Psomaglobin; PTL-78419; Pyrexol; pyriferone; Retrogen; Retropep; RG-003; Rhinostat; rifamaxil; RM-06; Rollin; romurtide; RU-40555; RU-41821; *Rubella* antibodies, ResCo; S-27609; SB-73; SDZ-280-636; SDZ-MRL-953; SK&F-107647; SL04; SL05; SM-4333; Solutein; SRI-62-834; SRL-172; ST-570;

ST-789; staphage lysate; Stimulon; suppressin; T-150R1; T-LCEF; tabilautide; temurtide; Theradigm-HBV; Theradigm-HPV; Theradigm-HSV; THF, Pharm & Upjohn; THF, Yeda; thymalfasin; thymic hormone fractions; thymocartin; thymolymphotropin; thymopentin; thymopentin analogues; thymopentin, Peptech; thymosin fraction 5, Alpha; thymostimulin; thymotrinan; TMD-232; TO-115; transfer factor, Viragen; tuftsin, Selavo; ubenimex; Ulsastat; ANGG-; CD-4+; Collag+; COLSF+; COM+; DA-A+; GAST-; GF-TH+; GP-120-; IF+; IF-A+; IF-A-2+; IF-B+; IF-G+; IF-G-1B+; IL-2+; IL-12+; IL-15+; IM+; LHRH-; LIPCOR+; LYM-B+; LYM-NK+; LYM-T+; OPI+; PEP+; PHG-MA+; RNA-SYN-; SY-CW-; TH-A-1+; TH-5+; TNF+; and UN.

Representative nucleoside and nucleotide compounds useful in the present invention include, but are not limited to:

(+) -cis-5-fluoro-l-(2- (hydroxy-methyl) -(1, 3-oxathiolan -5yl)cytosine;

(-) -2'-deoxy-3'-thiocytidine-5'-triphosphate (3TC) ;

(-) -cis-5-fluoro-l-(2- (hydroxy-methyl) -(1, 3-oxathiolan-5-yl)cytosine (FTC) ;

(-) 2', 3', dideoxy-3'-thiacytidine ((-) -SddC);

1- (2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) -5-iodocytosine (FIAC) ;

1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) -5-iodocytosine triphosphate (FIACTP) ;

1- (2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) -5-methyluracil (FMAU) ;

1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide;

2', 3'-dideoxy-3'-fluoro-5-methyl-dexocytidine (FddMeCyt) ;

2', 3'-dideoxy-3'-chloro-5-methyl-dexocytidine (C1ddMeCyt) ;

2', 3'-dideoxy-3'-amino-5-methyl-dexocytidine (AddMeCyt) ;

2', 3'-dideoxy-3'-fluoro-5-methyl-cytidine (FddMeCyt) ;

2', 3'-dideoxy-3'-chloro-5-methyl-cytidine (C1ddMeCyt) ;

2', 3'-dideoxy-3'-amino-5-methyl-cytidine (AddMeCyt) ;

2', 3'-dideoxy-3'-fluorothymidine (FddThd) ;

2', 3'-dideoxy-beta-L-5-fluorocytidine (beta-L-FddC) ;

2', 3'-dideoxy-beta-L-5-thiacytidine;

2', 3'-dideoxy-beta-L-5-cytidine (beta-L-ddC) ;

9- (1, 3-dihydroxy-2-propoxymethyl) guanine;

2'-deoxy-3'-thia-5-fluorocytosine;

3'-amino-5-methyl-dexocytidine (AddMeCyt) ;

2-amino-1, 9-((2-hydroxymethyl-l- (hydroxymethyl) ethoxy) methyl)-6H-purin-6-one (gancyclovir) ;

2-(2- (2-amino-9H-purin-9y) ethyl)-1, 3-propandil diacetate (famciclovir) ;

2-amino-1, 9-dihydro-9-((2-hydroxy-ethoxy) methyl)6H-purin-6-one (acyclovir) ;

2-amino-9-[2-[bis(2,2,2-trifluoroethyl)phosphonylmethoxy]ethyl]-6-p-methoxyphenylthiopurine (U.S. Patent No. 5,840,716);

5-azauridine ;

2-thio-6-azauridine ;

6-azauridine ;

5'-chloro-2',5'-dideoxy-2',2'-difluorocytidine ;

9- (4-hydroxy-3-hydroxymethyl-but-1-yl) guanine (penciclovir) ;

9- (4-hydroxy-3-hydroxymethyl-but-1-yl) -6-deoxy-guanine diacetate (famciclovir) ;

3'-azido-3'-deoxythymidine (AZT) ;

3'-chloro-5-methyl-dexocytidine (C1ddMeCyt) ;

9- (2-phosphonyl-methoxyethyl) –2', 6'-diaminopurine-2', 3'-dideoxyriboside;

9- (2-phosphonylmethoxyethyl) adenine (PMEA) ;

acyclovir triphosphate (ACVTP) ;

D-carbocyclic-2'-deoxyguanosine (CdG) ;

dideoxycytidine;

dideoxycytosine (ddC) ;

dideoxyguanine (ddG) ;

dideoxyinosine (ddI) ;

E-5- (2-bromovinyl) -2'-deoxyuridine triphosphate;

fluoro-arabinofuranosyl-iodouracil;

2',2'-difluorodeoxycytidine (gemcitabine);

1- (2'-deoxy-2'-fluoro-l-beta-D-arabinofuranosyl) -5-iodo-uracil (FIAU) ;

stavudine;

9-beta-D-arabinofuranosyl-9H-purine-6-amine monohydrate (Ara-A) ;

9-beta-D-arabinofuranosyl-9H-purine-6-amine-5'-monophosphate monohydrate (Ara-AMP) ;

2-deoxy-3'-thia-5-fluorocytidine;

2', 3'-dideoxy-guanine; and

2', 3'-dideoxy-guanosine.

Synthetic methods for the preparation of nucleosides and nucleotides useful in the present invention are well known in the art as disclosed in *Acta Biochim. Pol.*, 43:25-36 (1996); *Swed. Nucleosides Nucleotides* 15:361-378 (1996); *Synthesis* 12:1465-1479 (1995); *Carbohyd. Chem.* 27:242-276 (1995); *Chem. Nucleosides Nucleotides* 3:421-535 (1994); *Ann. Reports in Med. Chem.*, Academic Press; and *Exp. Opin. Invest. Drugs* 4: 95-115 (1995).

The chemical reactions described in the references cited above are generally disclosed in terms of their broadest application to the preparation of the compounds useful in the present invention. Occasionally, the reactions may not be applicable as described to each compound included within the scope of compounds disclosed herein. The compounds for which this occurs will be readily recognized by those skilled in the art. In all such cases, either the reactions can be successfully performed by conventional modifications known to those skilled in the art, e.g., by appropriate protection of interfering groups, by changing to alternative conventional reagents, by routine modification of reaction conditions, and the like, or other reactions disclosed herein or otherwise conventional will be applicable to the preparation of the corresponding compounds of this invention. In all preparative methods, all starting materials are known or readily preparable from known starting materials.

While nucleoside analogs are generally employed as antiviral agents as is, nucleotides (nucleoside phosphates) must sometimes have to be converted to nucleosides in order to facilitate their transport across cell membranes. An example of a chemically modified nucleotide capable of entering cells is S-1-3-hydroxy-2-phosphonyl-methoxypropyl cytosine (HPMPC, Gilead Sciences). Nucleoside and nucleotide compounds useful in the present invention that are acids can form salts. Examples include salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium, or magnesium, or with organic bases or basic quaternary ammonium salts.

Immunomodulators and immunostimulants useful in the combination therapy methods of the present invention can be administered in amounts equal to or lower than those conventional in the art. For example, interferon alpha is typically administered to humans for the treatment of hepatitis infections in an amount of from about 1 x 10^6

units/person three times per week to about 10 x 10^6 units/person three times per week (Simon et al., *Hepatology* 25: 445-448 (1997)). In the methods and compositions of the present invention, this dose can be in the range of from about 0. 1 x 10^6 units/person three times per week to about 7. 5 x 10^6 units/person three times per week; more preferably from about 0. 5 x 10^6 units/person three times per week to about 5 x 10^6 units/person three times per week; most preferably from about 1 x 10^6 units/person three times per week to about 3 x 10^6 units/person three times per week. Due to the anticipated enhanced hepatitis antiviral effectiveness of immunomodulators and immunostimulants in the presence of the anti-hepatitis dsRNA inhibitors of the present invention, it is expected that reduced amounts of these immunomodulators/immunostimulants can be employed in the methods and compositions disclosed herein. Such reduced amounts can be determined by routine monitoring of virus titers in infected patients undergoing therapy. This can be carried out by, for example, monitoring HCV RNA in patients' serum by slot-blot, dot-blot, or RT-PCR techniques such as the Amplicor® HCV Test, version 2.0, or the Cobas Amplicor™ HCV Test, version 2.0 (Roche Diagnostics Corp., Indianapolis, IN), or by measurement of HCV surface or other antigens. Patients can be similarly monitored during combination therapy employing the antiviral dsRNA inhibitors disclosed herein and other compounds having antiviral activity, for example nucleoside and/or nucleotide antiviral agents, to determine the lowest effective doses of each when used in combination.

In the methods of combination therapy disclosed herein, nucleoside or nucleotide antiviral compounds, or mixtures thereof, can be administered to human or animal patients in an amount in the range of from about 0.1 mg/patient/day to about 500 mg/ patient /day; preferably from about 10 mg/ patient /day to about 300 mg/ patient /day; more preferably from about 25 mg/ patient /day to about 200 mg/ patient /day; even more preferably from about 50 mg/ patient /day to about 150 mg/ patient /day; and most preferably in the range of from about 1 mg/ patient /day to about 50 mg/ patient /day.

Doses of compounds can be administered to a patient in a single dose or in proportionate multiple subdoses. In the latter case, dosage unit compositions can contain such amounts of submultiples thereof to make up the daily dose. Multiple doses per day can also increase the total daily dose should this be desired by the practitioner prescribing the drug.

The regimen for treating a patient suffering from an HCV infection with the dsRNA compounds and/or compositions of the present invention in the methods of combination therapy disclosed herein is selected in accordance with a variety of factors, including the age, weight, sex, diet, and medical condition of the patient, genotype of the infecting virus, the severity of the infection, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic, and toxicology profiles of the particular compounds employed, and whether a drug delivery system is utilized. Administration of the drug combinations disclosed herein should generally be continued over a period of several weeks to several months or years until virus titers reach acceptable levels, indicating that infection has been controlled or eradicated. Patients undergoing treatment with the individual dsRNA drugs or drug combinations disclosed herein can be routinely monitored by measuring virus viral RNA in patients' serum by slot-blot, dot-blot, or RT-PCR techniques, or by measurement of virus antigens, such as surface antigens, in serum to determine the effectiveness of therapy. Patient liver enzymes, such as alanine aminotransferase (ALT) or aspartate aminotransferase (AST), can also be monitored. Continuous analysis of the data obtained by these methods permits modification of the treatment regimen during therapy so that optimal amounts of each component used in monotherapy or combination therapy are administered, and so that the duration of treatment can be determined as well. Thus, the treatment regimen/dosing schedule can be rationally modified over the course of therapy so that the lowest amounts of each of the presently disclosed antiviral compounds used alone or in combination which exhibit satisfactory antiviral effectiveness are administered, and so that administration of such antiviral compounds is continued only so long as is necessary to successfully treat the infection.

The present invention encompasses the use of the antiviral dsRNA inhibitors disclosed herein alone, in combinations with one another, and in various combinations with the above-mentioned and similar types of compounds having antiviral activity, to treat or prevent HCV infections in patients. For example, one or more antiviral dsRNA inhibitors can be used in combination with: one or more interferons or interferon derivatives having antiviral activity; one or more non-interferon compounds having antiviral activity; or one or more interferons or interferon derivatives having antiviral activity and one or more non-interferon compounds having antiviral activity. When used

in combination to treat or prevent HCV infections in human or animal patients, any of the presently disclosed antiviral dsRNA inhibitors and above-mentioned compounds having antiviral activity can be present in a pharmaceutically or antiviral effective amount. By virtue of their additive or potentially synergistic effects, when used in the combinations described above, each can also be present in a subclinical pharmaceutically effective or antiviral effective amount, i.e., an amount that, if used alone, provides reduced pharmaceutical effectiveness in completely inhibiting or reducing the accumulation of HCV virions and/or reducing or ameliorating symptoms, conditions, or disorders associated with HCV infection or pathogenesis in patients compared to such antiviral dsRNA inhibitors and compounds having antiviral activity when used in pharmaceutically effective amounts. In addition, the present invention encompasses the use of combinations of antiviral dsRNA inhibitors and compounds having antiviral activity as described above to treat or prevent viral infections, where one or more of these antiviral dsRNA inhibitors or compounds is present in a pharmaceutically effective amount, and the other(s) is(are) present in a subclinical pharmaceutically effective or antiviral effective amount(s) owing to their additive or potentially synergistic effects. As used herein, the term "additive effect" describes the combined effect of two (or more) pharmaceutically active agents that is equal to the sum of the effect of each agent administered alone. A syngergistic effect is one in which the combined effect of two (or more) pharmaceutically active agents is greater than the sum of the effect of each agent administered alone.

Kits and Pharmaceutical Packs

For use in the therapeutic methods disclosed herein, the present invention also provides in one of its aspects a kit or pharmaceutical pack containing as a component one or more of the presently disclosed dsRNAs necessary to carry out the *in vitro* or *in vivo* introduction of the dsRNA to test samples or patients. In addition to a dsRNA, the kit or pack can contain a vehicle that promotes introduction of the dsRNA into a cell or patient. Such a kit may also include instructions to allow a user of the kit to practice the invention. The components can be in the form of a sterile-filled vial or ampoule containing one or more dsRNAs or a functionally equivalent variant thereof, or a vector encoding the same, operatively linked to regulatory sequences for expression, as well as instructions for use.

The vector can optionally be contained within a vector-releasing cell. In one embodiment, the kit contains a polynucleotide vector containing a dsRNA coding region, or functionally equivalent variant thereof, operatively linked to expression signals as an administration-ready formulation, in either unit dose or multi-dose amounts, wherein the package incorporates a label or manual with instructions for use of its contents for the treatment of a pathogen infection. In another embodiment, the package provides a sterile-filled vial or ampoule containing a vector-releasing cell or cell line. Such kits or packages can also contain media and reagents, such as reaction buffers, for carrying out appropriate methods as disclosed herein with the nucleic acids, recombinant constructs, vectors, or cells contained therein, as well as instructions therefor.

The following examples are provided purely to illustrate various aspects of the present invention, and should not be construed to be limiting thereof in any way.

Example 1

Inhibition of Hepatitis C Virus RNA Accumulation in Replicon Cells by Short Double Stranded RNA Oligonucleotides

Current standard therapy for HCV infection is treatment with the immunomodulator alpha-interferon (Chronic Hepatitis C: Current Disease Management, U.S. Department of Health and Human Services, National Institutes of Health, 1999). This therapy is ineffective in most HCV patients, who show either no response or a relapse even after prolonged interferon therapy. Additionally, there are severe side effects associated with interferon therapy.

In view of the pressing need for new, more effective antiviral drugs to treat HCV infected patients, the present inventors designed the dsRNA compounds described above. This example discloses the ability of various of these dsRNAs to inhibit the accumulation of HCV RNA in an HCV subgenomic RNA replicon assay (Replicon Assay) to determine their potential as anti-HCV therapeutics. The Replicon Assay is employed as a surrogate model for *in vitro* HCV infection to evaluate the effects of these dsRNAs, permitting measurement of the amount of HCV subgenomic RNA (replicon RNA) remaining in replicon cells (Lohmann *et al. Science* 285:110-113 (1999)) after dsRNA treatment relative to the amount of replicon RNA in untreated cells. In this assay, the potency of dsRNA compounds as HCV antiviral drugs is directly proportional to the level of

inhibition of replicon RNA accumulation. As shown below, the results demonstrate that there is a clear anti-HCV effect of dsRNAs of the present invention as analyzed by their capacity to reduce HCV RNA levels in the Replicon Assay.

The Replicon Assay

The Replicon Assay employing a cell line containing the self-replicating HCV subgenomic RNA (replicon) is described in Lohmann et al., *Science* 285:110-113 (1999). The GenBank accession number for the sequence of the replicon used in the experiments described herein is listed in this reference as AJ242654. This paper discloses methods for *in vitro* transcription of RNA from the replicon cDNA, transfection of the replicon RNA into Huh7 cells by electroporation, and selection of cells containing the replicon RNA using the antibiotic G418. Huh7 cells are a hepatoma cell line obtained from Dr. William Mason at Fox Chase Cancer Research Center (Philadelphia). These cells are publicly available from Fox Chase, and have been extensively described in the scientific literature (Nakabayashi *et al. Cancer Res.* 42:3858-3863 (1982)). In the experiments described herein, all of the template DNA is removed from the *in vitro* transcribed replicon RNA preparation prior to electroporation of this RNA into Huh7 cells by multiple treatment with DNase (three sequential treatments).

The replicon assay is performed as described in detail below. Briefly, cells containing the HCV replicon are placed in 96-well trays at a density of 10,000 cells per well and incubated in DMEM (Dulbecco's Minimal Essential Medium) supplemented with 10% fetal bovine serum, glutamine, nonessential amino acids, and the antibiotic G418 (0.25 mg/ml) at 37°C. After overnight incubation, the medium is replaced with OPTI-MEM (Invitrogen), which contains an HCV specific dsRNA oligonucleotide (667 nM) complexed with the transfection reagent Oligofectamine (Invitrogen), and the plates are incubated at 37°C for 4 hours. After a 48-hour incubation with the dsRNA oligonucleotides, the cells are collected by removing the medium and total cellular RNA is then extracted from the cells using the RNeasy-96 kit (Qiagen Inc., Valencia, CA). The amount of RNA isolated is then analyzed by quantitative RT-PCR, or TaqMan® (Applied Biosystems, Foster City, CA). The TaqMan® RT-PCR detects the RNA transcript derived from the neomycin resistance gene that is part of the replicon RNA.

For accurate quantitations, each treatment is carried out in five replicates, and untreated controls are carried out in 20 replicates.

Design and Synthesis of dsRNAs Homologous to Regions of the HCV Genome

All dsRNA oligonucleotides designed for testing as inhibitors of HCV infection, replication, and/or pathogenesis, including inhibition of HCV gene expression, comprise 19 nucleotide duplexes corresponding to various regions of the HCV genome, as well as two additional 2'-deoxythymidine nucleotides located at the 3'-terminus of each duplex strand. The G+C content of each dsRNA is approximately 40-60%. The nucleotide sequences of the dsRNAs are shown in the Table 1, below. The dsRNAs are synthesized *in vitro* using 2'-ACE chemistry (Dharmacon Technical Bulletin #003, 2001). The dsRNAs are synthesized as single stranded RNA oligonucleotides, deprotected, desalted, and then annealed as suggested by the supplier (Dharmacon Technical Bulletin #003, 2001). The dsRNAs are dissolved in DEPC-treated distilled H_2O to a final concentration of 20 μM, and stored at -80°C. As controls, the following dsRNAs are also synthesized as described above: LZNeo1, corresponding to the neomycin resistance gene, and LZR19, corresponding to a portion of the human rhinovirus isolate 16.

TABLE 1

RNA Sequences of dsRNAs	
dsRNA designation	**dsRNA sequence**
LZNeo1	5' AUGGAUUGCACGCAGGUUCTT 3' 3'TTUACCUAACGUGCGUCCAAG 5'
LZR19	5' GATGGCTTGGAAGCTTTGGTT 3' 3'TTCTACCGAACCTTCGAAACC 5'
LZ107	5' GACCUUCACCAUUGAGACGTT 3' 3'TTCUGGAAGUGGUAACUCUGC 5'
LZ121	5' GGGUGUCCAUUGCCGCCUGTT 3' 3'TTCCCACAGGUAACGGCGGAC 5'
LZ123	5' CAGGCGCCCUGAUCACGCCTT 3' 3'TTGUCCGCGGGACUAGUGCGG 5'
LZ125	5' GACACCAAUUGACACCACCTT 3' 3'TTCUGUGGUUAACUGUGGUGG 5'
LZ129	5' CGCGCACGAUGCAUCUGGCTT 3' 3'TTGCGCGUGCUACGUAGACCG 5'
LZ133	5' AGUGGAUGAACCGGCUGAUTT 3' 3'TTUCACCUACUUGGCCGACUA 5'
LZ135	5' UCACGGAGGCUAUGACUAGTT 3' 3'TTAGUGCCUCCGAUACUGAUC 5'

Details of the Replicon Assay are as follows.

Procedure for Quantitative Analysis of HCV Replicon RNA in the HCV Replicon Assay Using TaqMan® RT-PCR

The Replicon Assay is used to measure the capacity of potential HCV antiviral compounds to inhibit the accumulation of an HCV subgenomic RNA replicon molecule in a Huh7 cell line (Lohmann et al., *Science* 285:110-113 (1999)). This assay comprises three operational components : (1) Replicon cell maintenance, assay plate set up, and compound application; (2) Extraction of total cellular RNA from replicon cells; and (3) Real time RT-PCR (TaqMan®) to measure the mount of replicon RNA in each sample. The Replicon Assay requires at least four days to perform; however, the process can be interrupted and samples frozen between steps. Each assay component is described below.

1. Replicon cell maintenance, assay plate setup, and compound application

1.1 Replicon cell line maintenance

The cell line used in the Replicon Assay is produced as described in Lohmann et al. (*Science* 285:110-113 (1999)). After 150 cm^2 cell culture flasks (Costar) containing Replicon cells are incubated at 37°C under 5% CO_2 and become confluent, the cells are

diluted 1:10, v/v, into fresh 150 cm^2 cell culture flasks. The medium is DMEM containing 10% fetal bovine serum (FBS), 1X non-essential amino acids (NEAA), 1X Glutamine (Glu), and 0.25 mg/ml G418. Three serial passages are performed, each time allowing the cells to become confluent, followed by dilution of the cells into fresh 150 cm^2 cell culture flasks. These cells, referred to as "original cells," are then aliquoted and stored for future use in the Replicon Assay. TaqMan®-based analysis is performed to determine the number of HCV replicon genomes per cell, which reveals the presence of ~150 to 1,000 copies of the replicon per cell. This is based on the ratio of copies of replicon RNA to two times the copies of the human *apo*B gene (number of haploid genomes).

1.1.1 Original cells are stored in liquid N_2. For cells used in the Replicon Assay, after 20 serial passages, cells are abandoned, and a fresh lot is revived from liquid N_2 storage.

1.2 Plating of cells in 96-well trays for the Replicon Assay

1.2.1 For preparation of 96-well plates, a 75% confluent 75 cm^2 flask of replicon-containing cells is trypsinized and resuspended in 10 ml Medium A (Table 3). Trypsinization is performed by removing the medium, adding 1 ml of trypsin-EDTA 0.25%, w/v, and then removing the trypsin-EDTA. After 5-10 minutes, the cells release from the flask and are resuspended in medium A.

1.2.2 Cells are counted using a hemacytometer, and the cell concentration is adjusted to 10^5 cells/ ml.

1.2.3 Each well is seeded with a 100 µl cell suspension using an Impact2 multi-channel pipette (Matrix), never plating more than four 96-well plates from a single cell suspension.

1.2.4 96-well plates are incubated at 37°C under 5% CO_2 overnight.

1.3. Testing dsRNAs in the HCV Replicon Assay

1.3.1 Anti-HCV dsRNAs are dissolved in distilled H_2O to a final concentration of 20 µM.

1.3.2 The 20 μM dsRNAs are diluted in OPTI-MEM to a concentration of 741 nM (1 μl of 20 μM solution to 26 μl of OPTI-MEM). The diluted dsRNAs are incubated at room temperature for 5 minutes.

1.3.3 0.5 μl of the transfection reagent, Oligofectamine, is diluted with 2.5 μl of OPTI-MEM. The diluted Oligofectamine is incubated for 5 minutes at room temperature.

1.3.4 The diluted dsRNAs from step 1.3.2 and the diluted Oligofectamine from step 1.3.3 are mixed and incubated at room temperature for at least 15 minutes. The final concentrations of dsRNAs in the transfection mixtures are 667 nM.

1.3.5 The plates containing cells are removed from the 37°C, 5% CO_2 incubator and labeled on the top right corner of the lid and the right side of the base. The medium is poured off of the 96-well plates.

1.3.6 The cells are washed with 150 μl of OPTI-MEM.

1.3.7 30 μl of the dsRNA transfection mix from step 1.3.4 are overlayed on the monolayer of the cells. Medium C (Table 3) is added to all the untreated wells according to Table 2 for testing dsRNAs. "Untx" refers to mock-treated cells with Oligofectamine added at the same concentration as in treated cells; "dsRNA" refers to a specific dsRNA at a concentration of 667 nM. "Con" refers to the concentration of a dsRNA. "Cells" refers to wells containing cells treated only with Medium C, i.e., not with Medium C containing Oligofectamine or Oligofectamine plus dsRNA.

TABLE 2

Ten dsRNAs, One Concentration, Five Replicates

	1	2	3	4	5	6	7	8	9	10	11	12
A	untx	untx	untx	untx	untx	untx	untx	untx	untx	untx		
B	untx	untx	untx	untx	untx	untx	untx	untx	untx	untx		
C	dsRNA 1	dsRNA 2	dsRNA 3	dsRNA 4	dsRNA 5	dsRNA 6	dsRNA 7	dsRNA 8	dsRNA 9	dsRNA 10		
D	dsRNA 1	dsRNA 2	dsRNA 3	dsRNA 4	dsRNA 5	dsRNA 6	dsRNA 7	dsRNA 8	dsRNA 9	dsRNA 10		
E	dsRNA 1	dsRNA 2	dsRNA 3	dsRNA 4	dsRNA 5	dsRNA 6	dsRNA 7	dsRNA 8	dsRNA 9	dsRNA 10		
F	dsRNA 1	dsRNA 2	dsRNA 3	dsRNA 4	dsRNA 5	dsRNA 6	dsRNA 7	dsRNA 8	dsRNA 9	dsRNA 10		
G	dsRNA 1	dsRNA 2	dsRNA 3	dsRNA 4	dsRNA 5	dsRNA 6	dsRNA 7	dsRNA 8	dsRNA 9	dsRNA 10		
H	cells	cells	Cells	cells	cells	cells	cells	cells	cells	cells		

2 dsRNAs, 5 concentrations, 5 replicates

	dsRNA 1					dsRNA 2						
	1	2	3	4	5	6	7	8	9	10	11	12
A	untx	untx	Untx	untx	untx	untx	untx	untx	untx	untx		
B	untx	untx	Untx	untx	untx	untx	untx	untx	untx	untx		
C	Con1	Con2	Con3	Con4	Con5	Con1	Con2	Con3	Con4	Con5		
D	Con1	Con2	Con3	Con4	Con5	Con1	Con2	Con3	Con4	Con5		
E	Con1	Con2	Con3	Con4	Con5	Con1	Con2	Con3	Con4	Con5		
F	Con1	Con2	Con3	Con4	Con5	Con1	Con2	Con3	Con4	Con5		
G	Con1	Con2	Con3	Con4	Con5	Con1	Con2	Con3	Con4	Con5		
H	cells	cells	Cells	cells	cells	cells	cells	cells	cells	cells		

1.4. The plates are incubated for 48 hours at 37°C under 5% CO_2, and then subjected to RNA extraction.

TABLE 3

Summary of Equipment and Supplies for Cell Culture and dsRNA Set Up

Equipment and Supplies	Catalog. No.	Supplier
8 channel Impact2 Pipette, 1250 µl	Cat. no. 2004	Matrix
2 ml polypropylene deep-well block, 96-well, sterile	Cat. no. 4222	Matrix
25 ml Reagent Reservoirs, Sterile	Cat. no. 8096	Matrix
1250 µl X-tra long pipet tips	Cat. no. 8255	Matrix
96-well plate	Cat. no. 3595	Costar
Hemacytometer	Bright line improved Neubauer 0.1 mm deep	Reichert
DMEM	Cat. no. 51444-79P	JRH
L-glutamine (Glu)	Cat. no. 12403-010	GIBCO-BRL
Non-essential amino acids (NEAA)	Cat. no. 11140-050	GIBCO-BRL
Fetal Bovine Serum (FBS)	Cat. no. 16250-078	GIBCO-BRL
Oligofectamine	Cat. no. 12252-011	Invitrogen
G418	Cat. no. 55-0273	Invitrogen
Medium A	DMEM, 10% FBS, 1X NEAA, 1X Glu, 0.25 mg/ml G418	
Medium B	DMEM, 2% FBS, 1X NEAA, 1X Glu	
Medium C	DMEM, 2% FBS, 1X NEAA, 1X Glu, 0.5 µl Oligofectamine (per well)	
Trypsin-EDTA 0.25%	GIBCO-BRL	

2. Extraction of total cellular RNA from replicon cells

2.1 Introduction

The goal of this procedure is to extract RNA from *in vitro* tissue culture samples so that the viral or cellular RNA is quantitatively recovered and pure enough to be analyzed by quantitative HCV RT-PCR assay.

To permit detection of variations in the efficiency of the RNA extraction, standard amounts of bovine viral diarrhea virus (BVDV), an RNA virus with some similarity to HCV, are added to each cell sample before RNA extraction. Thus, the level of BVDV RNA detected in the final multiplex RT-PCR reaction should be consistent among all wells within the variability limits associated with the Replicon Assay. This RNA extraction efficiency internal control is discussed further in the TaqMan® section, below.

The RNA extraction procedure used is the RNeasy-96 method supplied by Qiagen Inc. (Valencia, CA). This method employs 96 silica-based mini-columns that are positioned in an array compatible with 96-well tissue culture operations. The RNA extraction technology is a modification of the Boom method, in which all cellular proteins and nucleic acid, including nucleases, are first denatured with a strong chaotropic salt (guanidinium thiocyanate). In this environment, nucleic acids have a strong affinity for silica, the material in the mini-column discs; however, proteins and other contaminants do not bind to silica, and pass through the columns. After washing the columns with chaotropic/ethanol solutions, the samples are partially dried, and the nucleic acid is then released from the column in a small volume of water.

To reduce variability in recovering HCV RNA, care is taken with the column washing and partial drying conditions. The presence of a small amount of ethanol on a column will contaminate the final RNA and interfere with the RT-PCR detection system. Caution is required in all phases of this procedure because the starting samples may be biohazardous, the chaotropic salt is highly caustic, and as a thiocyanate, it can generate poisonous cyanide gas if allowed to come in contact with acidic environments.

TABLE 4

Summary of Equipment and Supplies for HCV RNA Extraction Procedures		
Equipment and Supplies	**Catalog No.**	**Supplier**
RNeasy 96 Kit (24)	Cat. no. 74183	Qiagen
QIAvac 96 manifold	Cat. no. 19504	Qiagen
Centrifuge 4-15C, for 2x96 plates, 6000 x g	Cat. no. 81010	Qiagen
plate rotor for 2x96 plates	Cat. no. 81031	Qiagen
200 Proof Ethyl Alcohol		
8 channel Impact2 Pipette, 250 µl	Cat. no. 2002	Matrix
8 channel Impact2 Pipette, 1250 µl	Cat. no. 2004	Matrix
2 ml polypropylene deep-well block, 96-well, sterile	Cat. no. 4222	Matrix
25 ml Reagent Reservoirs, Sterile	Cat. no. 8096	Matrix
1250 µl X-tra long pipet tips	Cat. no. 8255	Matrix
200 µl pipet tips	Cat. no. 7275	Matrix
serum free MEM medium	Cat. no.11095-80	GIBCO-BRL

2.2 Procedure:

2.2.1 Cell Lysis

2.2.1.1 Prepare lysis buffer: For one 96-well plate, add 150 µl β-mercaptoethanol (β-ME) and 1 µl BVDV stock (vortex stock before adding) to 15 ml RLT buffer (a component of the RNeasy kit, Qiagen). This stock is prepared by infecting MDBK cells (bovine kidney cells , #CCL-22, available from the American Type Culture Collection, Manassas VA) with BVDV and harvesting the culture at peak cytopathic effect (CPE). This stock has an infectious titer of approximately $1x10^7$ pfu/ml. This gives BVDV a threshold cycle (C_t) of about 22 in the TaqMan® assay. The BVDV stock is stored in a -80°C freezer.

2.2.1.2 Cells are washed with 150 µl serum-free MEM medium (program 4 on 8 channel electronic pipette P1250: Fill 1250, Disp 150 x 8). 150 µl lysis buffer are added to each well (same program).

2.2.1.3 RNA is extracted immediately, or cells are frozen at -80 °C.

2.2.2 Preparation of reagents and materials for RNA extraction.

2.2.2.1 Note the lot number of the RPE and RNeasy 96 Kit.

2.2.2.2 RPE: 720 ml of 100% ethanol are added to one bottle of RPE (Qiagen), and mixed well; RPE bottles are always shaken well before use.

2.2.2.3 70% Ethanol: 150 ml diethylpyrocarbonate (DEPC) water are added to 350 ml 100% ethanol and mixed well.

2.2.3 Preparation of RNA with RNeasy 96 kit

2.2.3.1 Frozen samples are thawed at room temperature for 40 min. At the same time, one column of Extraction Controls is thawed for each plate (Extraction Controls: The RNeasy Extraction Controls are a set of 8 tubes all connected together. Inside of each tube is 170 µl of cell lysate with a certain ratio of HCV positive and negative cells. From the top to the bottom are two each of a low, medium, high, and zero number controls, respectively. (See section 2.3 of the protocol below).

2.2.3.2 The samples are mixed by pipetting 100 µl up and down five times. The entire sample is transferred into columns 1-10 of the 2 ml Matrix square-well block (program 1 on P250: Mix 100 x 5, Fill 170, Purge).

2.2.3.3 150 µl of the replicon standard is transferred into column 11(no samples in column 12).

2.2.3.4 150 µl of 70% ethanol (EtOH) are added to each sample (program 4 on P1250: Fill 1250, Disp 150).

2.2.3.5 An RNeasy 96 plate labelled with the appropriate plate number is placed in the vacuum manifold. Mix and transfer the lysate/EtOH to the RNeasy 96 plate (program 1 on P1250: Mix 200, Times 5, Fill 330, and Purge). Any unused wells are sealed with transparent tape (supplied by Qiagen), usually column 12.

2.2.3.6 Vacuum (approximately 800 mbar) is applied to load the sample onto the mini-columns.

2.2.3.7 The RNeasy-96 plate is washed with 1000 µl of RW1 buffer (Qiagen) per well (program 2 on P1250: Fill 1000, Disp 1000).

2.2.3.8 Vacuum is applied to the filter through the RW1 buffer, and the flow-through is emptied.

2.2.3.9 The RNeasy-96 plate is washed with 1000 µl of RPE buffer per well (program 2 on P1250).

2.2.3.10 Vacuum is applied to filter through the RPE buffer.

2.2.3.11 Repeat Step 2.2.3.9

2.2.3.12 Vacuum is applied to the filter through the RPE buffer, keeping the vacuum applied for 3 min.

2.2.3.13 Dry the RNeasy 96 plate: The RNeasy-96 plate is placed in a collection microtube rack (supplied by Qiagen), covered with the supplied AirPore tape, and the unit is centrifuged for 10 min at 6000 x g (Qiagen 4-15C centrifuge).

2.2.3.15 Elute the RNA from the RNeasy 96-well plate: The RNeasy-96 plate is transferred onto the top of a new collection microtube rack. 70 µl of RNase-free water are added to the middle of each well (program 3 on P1250: Fill 850, Disp 70).

2.2.3.16 Incubate 1 min at room temperature, and then place a fresh AirPore tape over the plate.

2.2.3.17 The unit is then centrifuged for 4 min at 6000 x g in a Qiagen 4-15C centrifuge. The eluted volume measures between 28 µl and 50 µl.

2.2.3.18 The RNeasy-96 plate is discarded, and the collection tube rack is sealed with the Qiagen-provided caps (8 per strip).

2.2.3.19 The eluted RNA is stored at –80°C or immediately analyzed in the TaqMan® assay.

2.3 Extraction Controls Preparation

Day 1

2.3.1.1 Plate out 2.5 x 10^7 replicon-producing cells in a 150 cm^2 tissue culture flask (T-150).

2.3.1.2 Plate out 2.0 x 10^6 Huh7 cells in a 75 cm^2 tissue culture flask (T-75).

2.3.1.3 Incubate overnight at 37 °C under 5% CO_2.

Day 2

2.3.1.4 Lyse the cells with lysis buffer.

2.3.1.5 Remove the supernatant from the Huh7 and replicon-producing cells, and wash the monolayer with 10 ml serum-free medium (MEM).

2.3.1.6 Add 30 ml of lysis buffer (with 1 µl of BVDV stock/15 ml of lysis buffer) to the Huh7 cells, mix by repeated pipetting, and place the cell lysate in a 50 ml conical-bottomed tissue culture centrifuge tube.

2.3.1.7 Add 10.5 ml of lysis buffer to the replicon-producing cells, mix by repeated pipetting, and place the cell lysate in a 15 ml conical-bottomed tissue culture centrifuge tube.

2.3.2 For the HIGH Extraction Standard: Aliquot 170 µl of the replicon-producing cells cell lysate into rows 5 and 6 of two Matrix 0.75 ml tube racks.

2.3.3 For the MEDIUM Extraction Standard: Add 1.0 ml of the replicon-producing cells cell lysate to 9 ml of the Huh7 lysate, and mix well. Aliquot 170 µl of this mixture to rows 3 and 4 of two Matrix 0.75 ml tube racks.

2.3.4 For the LOW Extraction Standard: Add 50 µL of the replicon-producing cells cell lysate to 10 ml of the Huh7 lysate, and mix well. Aliquot 170 µl of this mixture to rows 1 and 2 of two Matrix 0.75 ml tube racks.

2.3.5 ZERO Extraction Control: Aliquot 170 µL of the Huh7 cell lysate to rows 7 and 8 of two Matrix 0.75 ml tube racks.

2.3.6 Store controls at –80 °C

3. TaqMan® RT-PCR and Data Analysis

3.1 Introduction: Real-time quantitative RT-PCR is used to measure the amount of HCV replicon RNA in each sample. This technology is also referred to as the PCR-based 5' nuclease assay, and TaqMan®. The analytic instrument is the Applied Biosystems 7700 Prism Sequence Detection System (Applied Biosystems, Foster City, CA). This instrument is essentially a time-multiplexed laser-induced fluorescence spectrograph coupled with a thermal cycler. It monitors the accumulation of PCR amplicon in each well of a 96-well sample tray throughout the course of the PCR process.

3.2. Use of BVDV Internal Control: As mentioned in the previous section, an internal positive control is incorporated into every sample. This serves as a measure of RNA extraction efficiency, and shows if the sample contains contaminants that inhibit TaqMan® PCR. BVDV is mixed with the chaotropic cell lysis buffer prior to applying the lysis buffer to the cells. Although the positive control is in every sample, the BVDV

internal positive control assay is only performed when the HCV replicon RNA assay data fall outside of expected limits, suggesting that there could be a problem with the samples. Specific criteria that elicit a TaqMan® analysis for the BVDV internal positive control of a sample plate are described in the section on data analysis (3.6).

3.3. HCV Replicon RNA TaqMan® probe and primers. Because of the expected genetic stability and general lack of RNA secondary structure in the neomycin resistance gene (*neo*) encoded in the replicon, primers and a probe that bind in that region are employed. This segment of the replicon RNA extends from bases 342-1193 of the 8001 base pair replicon:

```
301 gtgcttgcga gtgccccggg aggtctcgta gaccgtgcac catgagcacg aatcctaaac
361 ctcaaagaaa aaccaaacgt aacaccaacg ggcgcgccat gattgaacaa gatggattgc
421 acgcaggttc tccggccgct tgggtggaga ggctattcgg ctatgactgg gcacaacaga
481 caatcggctg ctctgatgcc gccgtgttcc ggctgtcagc gcaggggcgc ccggttcttt
541 ttgtcaagac cgacctgtcc ggtgccctga atgaactgca ggacgaggca gcgcggctat
601 cgtggctggc cacgacgggc gttccttgcg cagctgtgct cgacgttgtc actgaagcgg
661 gaagggactg gctgctattg ggcgaagtgc cggggcagga tctccctgtca tcttcacctg
721 ctcctgccga gaaagtatcc atcatggctg atgcaatgcg gcggctgcat acgcttgatc
781 cggctacctg cccattcgac caccaagcga aacatcgcat cgagcgagca cgtactcgga
841 tggaagccgg tcttgtcgat caggatgatc tggacgaaga gcatcagggg ctcgcgccag
901 ccgaactgtt cgccaggctc aaggcgcgca tgcccgacgg cgaggatctc gtcgtgaccc
961 atggcgatgc ctgcttgccg aatatcatgg tggaaaatgg CCGCTTTTCT GGATTCATCG
                                    forward primer
1021 aCTGTGGCCG GCTGGGTGTG Gcggaccgct atcaggacat agcgttggct acccgtgata
        TaqMan probe
1081 ttgctgaaga gcTTGGCGGC GAATGGGctg accgcttcct cgtgctttac ggtatcgccg
              reverse primer
1141 ctcccgattc gcagcgcatc gccttctatc gccttcttga cgagttcttc tgagtttaaa
```

3.4. Procedures

3.4.1. Method for Preparing 1x Master Mixtures for NEO and BVDV RT-PCR

TABLE 5

Equipment and Supplies for Preparing RT-PCR 10-Plate Master Mix

Equipment and Supplies	Catalog No.	Supplier
0.5-10 µl pipette	22 47 005-1 2000 Series	Eppendorf
2-20 µl pipette	22 47 015-9 2000 Series	Eppendorf
10-100 µl pipette	22 47 020-5 2000 Series	Eppendorf
50-200 µl pipette	22 47 025-6 2000 Series	Eppendorf
100-1000 µl pipette	22 47 030-2 2000 Series	Eppendorf
1250µl Matrix tips	Cat. no. 8255	Matrix
200 µl Matrix tips	Cat. no. 7275	Matrix
10 µl ART tips	Cat. no.2140	Molecular Bioproducts
20 µl ART tips	Cat. no.2149P	Molecular Bioproducts
100 µl ART tips	Cat. no.2065E	Molecular Bioproducts
200 µl ART tips	Cat. no.2069	Molecular Bioproducts
1000 µl ART tips	Cat. no.2079E	Molecular Bioproducts
Electronic pipette, Impact2	Cat. no. 2001	Matrix
1.5 ml RNase-free microfuge tubes	Cat. no. 12450	Ambion
14 ml Polypropylene tubes	Cat. no. 352059	Falcon
25 ml reagent reservoir	Cat. no. 8096	Matrix
96-well reaction plate	Cat. no. N801-0560	Applied Biosystems
optical cap strips	Cat. no. N801-0935	Applied Biosystems
Disposable Sterile Gowns	Cat. no. 9515-E	Baxter
Reagents	**Catalog No.**	**Supplier**
Acid	0.1N HCl	Fisher
RNAseZap	Cat. no. 9780	Ambion
RNAse away	Cat. no. 7005	Molecular Bioproducts
10-pak, EZ RT-PCR core reagents kit, 5x reaction buffer, 25 mM Manganese Acetate, deoxy NTPs	Cat. no. 403028	Applied Biosystems
VIC NEO probe, 2 µM (=10x), 550µl per aliquot	Cat. no. 450003, custom, 5'-VIC-CTG TGG CCG GCT GGG TGT GG-TAMRA -3'	Applied Biosystems

VIC BVDV probe, 2μM (=10x), 550 μl per aliquot (Vertex)	Cat. no. 450003, custom, 5'-VIC-CCC TCG TCC ACG TGG CAT CTC GA-TAMRA-3'	Applied Biosystems
NEO forward primer, 3μM (=10x) forward/reverse primer mix, 550 μl per aliquot	Cat. no. 4304972, custom, 5'-CCG CTT TTC TGG ATT CAT CG-3'	Applied Biosystems
NEO reverse primer, 3 μM (=10x) forward/reverse primer mix, 550 μl per aliquot	Cat. no. 4304972, custom, 5'-CCC ATT CGC CGC CAA-3'	Applied Biosystems
BVDV forward primer, 3 μM (=10x) forward/reverse primer mix, 550 μl per aliquot	custom, 5'-CAG GGT AGT CGT CAG TGG TTC G-3', 1.0 μM scale w/gel purification	Oligos etc
BVDV reverse primer, 3 μM (=10x) forward/reverse primer mix, 550 μl per aliquot	custom, 5'-GGC CTC TGC AGC ACC CTA TC-3', 1.0 μM scale w/ gel purification	Oligos etc
NEO RNA standards	*In vitro* transcribed RNA from a plasmid containing the neo gene portion of the HCV replicon RNA using T7 RNA polymerase. The *in vitro* transcribed RNA is quantitated based on known molecular weight of the transcripts and the UV-absorbance of the purified transcript solution. This RNA is diluted, aliquoted, and stored at -80°C. Individual aliquots are thawed for each TaqMan® assay.	
RNA samples to be tested isolated from HCV replicon cells (section 2 of this Protocol), 10 μl/96-well plate		
Nuclease-Free Water (Not DEPC Treated)	Cat. no. 9930	Ambion

3.4.2 Preparation of reagents for master mixture

3.4.2.1 Clean the bench according to the two steps below, and wipe the pipettes with RNAse away.

RNAse Zap (Ambion, Austin, TX)

RNAse Away (Molecular Bioproducts, San Diego, CA)

3.4.2.2 Open core EZ RT-PCR reagents (Applied Biosystems) and put the 5x buffer on ice, thaw frozen reagents at room temperature for about 15 minutes, and then put them on ice. One EZ RT-PCR reagents kit can be used to analyze two 96-well RNA extractions.

3.4.2.3 Take one tube of 2 μM VIC probe (NEO or BVDV, 550 μl per tube) from -20°C and put on ice.

3.4.2.4 Take one tube 3 μM forward/reverse primer mix (NEO or BVDV, 550 μl per tube) from –20°C and put on ice.

3.4.2.5 Take one tube (30 μl) of standards RNA transcript (10^8 copies/10 μl) from –80°C and place on ice.

3.4.2.6 Take one tube of room temperature Ambion water.

3.4.3 Assembly of master mixture for one 96-well plate reaction.

3.4.3.1 Use a 1ml pipette to transfer 5x buffer (Applied Biosystems) to a 14 ml tube; total volume added is 1100μl.

3.4.3.2 Use a 1ml pipette to add 25mM $Mn(OAc)_2$ (Applied Biosystems) to a 14 ml tube; total volume added is 660μl.

3.4.3.3 Use a 200μl pipette to add 165μl of 10 mM dATP to the 14ml tube. Do the same for 10mM dCTP, 20mM dUTP, and 10mM dGTP.

3.4.3.4 Use a 1 ml pipette to add 550 μl 10x 3 μM forward/reverse primer mix.

3.4.3.5 Use a 1 ml pipette to add 550 μl 10x 2 μM probe.

3.4.3.6 Use a 1 ml pipette to add 220 μl *rTth* DNA polymerase (Applied Biosystems).

3.4.3.7 Use a 100 μl pipette to add 55 μl AmpErase UNG (Applied Biosystems).

3.4.3.8 Use a 1 ml pipette to add 605 μl Ambion H_2O to the 14 ml tube; the final volume is 4400μl total.

3.4.3.9 Transfer the 4400 µl master mix to a 25 ml reagent reservoir.

3.4.3.10 Dispense 40 µl per well for all 96 wells using an 8-channel pipette.

3.4.3.11 Transfer 10 µl of extracted unknown samples to wells of the reaction plate using an 8-channel pipette, column by column, column 1 through column 11. Cap each column after transfer.

3.4.3.12 Add 270 µl Ambion H_2O to the 30 µl 10^8 copies/10 µl RNA transcript for use in the standard curve and mix. There are now 10^7 copies of the HCV replicon quantitation standard RNA/10 µl.

3.4.4 Setting up the ABI 7700 for each run

3.4.4.1 Before each run, reboot the computer for the ABI 7700 and rebuild the desktop.

3.4.4.2 Close and remove any redundant programs from the hard drive; overuns data to trash.

3.4.4.1 Open Sequence Detector v1.7 program (SDS software).

3.4.4.5 Open the "Replicon Assay Runs" folder.

3.4.4.6 Open the "Replicon Assay" template plate. The thermal cycler conditions programmed into the template are as follows:

Stage 1: 50°C for 2 min.

Stage 2: 60°C for 30 min.

Stage 3: 95°C for 5 min.

Stage 4: 95°C for 15 sec.

Stage 5: 60°C for 60 sec.

Cycle repeat number of stages 4-5: 40.

Template instrument:diagnosis: advanced options:

Select views: display mse.

Select views: display best fit.

Select miscellaneous: reference dye ROX.

3.4.4.7 "Save" (not "save as") the file in the "Replicon Assay Runs" folder.

3.4.4.8 Show setup: hit RUN

3.5 Preparing the ABI7700 data after a run using SDS software.

3.5.1 The assay plates are removed from the ABI7700 and discarded without ever being opened. This greatly reduces laboratory problems with PCR cross contamination.

3.5.2 The data are analyzed using the Sequence Detector System software V1.7.

3.5.3 The threshold levels are initially set using default settings.

3.5.4 Data rejection criteria: Data points or series of whole plates can be rejected. If there has been a significant deviation from protocol, reagent failure or mishap, or ABI 7700 run failure, data can be discarded. For rejection of any data points from an apparently normal run, one or more of these criteria must be met.

3.5.4.1 Threshold cycle calculations. Normally use the default values for the SDS software. If the Ct of the most concentrated sample is less than 15, then change the threshold value stop limit as needed to a lower value so that the Ct of the highest concentration sample is greater than the stop value. Update calculations after making this change.

3.5.4.2 Consider rejecting an entire abnormal TaqMan® run as indicated by a deviation from the mean values for the slope and y-axis intercept of the line generated by analysis of the *neo* RNA standards. The acceptable ranges for those values are:

Slope values should be between 3.0 and 3.6

y-intercept cycles should be between 36 and 41 cycles.

3.5.4.3 Aberrant individual TaqMan® wells as indicated by extreme Rn/ΔRn can be deleted prior to data analysis so that they do not affect the SDS software calculations.

3.5.4.4 Examine and record the no-template control Ct values and confirm that they are >7.0 Ct (>100X) higher than the Ct for any compound treated sample.

3.5.5 The HCV RNA standards Ct values are compared with previous results.

3.5.6. The HCV RNA standard curve is compared with previous results.

3.5.7 If aberrant amplification is evident in individual wells, those wells are identified and noted.

3.5.8 The "results" file is exported and transferred from the 7700 computer to another computer for analysis using Microsoft Excel.

3.5.9 Any of the following changes in reagent preparations or dilution used are reported.

New probe or primer synthesis from vendor.

New probe or primer dilution and aliquots.

New standards RNA transcript preparation.

New standards RNA transcript dilution and aliquots.

New BVDV viral preparation.

New column 11 standards preparation.

3.6 TaqMan® data analysis.

3.6.1 Copy and paste TaqMan® HCV Ct number and copy number from the TaqMan® results file into the appropriate cells of the Replicon Assay data analysis Microsoft Excel macro, and run the macro.

3.6.2 Copy the TaqMan® results table from the macro sheet onto another sheet, input compound serial number and lot number.

3.6.3 From this excel sheet, the mean, standard deviation, and percentage CV of Compound inhibition activity, as well as HCV copy number, HCV Ct number, and BVDV Ct number (if available), of all dilution points in 5 replicates and no-compound control, will be calculated.

3.6.4 Criteria for data rejection and implementation of BVDV Control TaqMan®. Check all the calculations. Data points or series of whole plates can be rejected. If there is a significant deviation from protocol, reagent failure or mishap, or ABI 7700 run failure, data can be discarded. For rejection of any data points from an apparently normal run, then one or more of these criteria must be met. The standard deviation of percentage inhibition should be less than 30% in active compounds. The %CV of HCV copy number should be less than 30%. The standard deviation of HCV Ct of all samples should be less than 0.5; this is usually about 0.1 to 0.3 in most samples. If the HCV Ct standard deviation is more than 0.5, then go back to the raw data table, and check the Ct numbers of 5 replicates. If the Ct number of any one well is 2 Ct different from the average Ct number of 5 replicates, then this well should omitted from the analysis. If more than 3 wells (not on same column) have unusual Ct numbers, then the

BVDV TaqMan® internal control assay should be carried out. If the BVDV data show irregularity, then the compound should be tested again.

3.6.5 IC_{50} calculation: Copy and paste the data of average inhibition and standard deviation into a sigmoid dose response with a variable slope calculator that uses non-linear regression methods. Using this tool, calculate the IC_{50} by using both of two methods: fixing the top at 100% inhibition only, or fixing the top at 100% inhibition and the bottom at 0% inhibition. The method that gives the clearest fit is then reported for each dsRNA compound. The most reliable IC_{50} comes from the calculation having the lowest standard error. If IC_{50}s calculated from these two curve fit options show more than one fold difference, or if the IC_{50} SD is greater than the IC_{50}, the dsRNA compound should be tested again at adjusted concentrations.

Results

To determine if dsRNA oligonucleotides corresponding to various regions of the HCV genome can inhibit HCV replicon replication as measured by quantatitive PCR, cells containing the HCV replicon are transfected with different dsRNA oligonucleotides using Oligofectamine as described above. After incubation of transfected cells for two days, the amount of RNA isolated from transfected and untransfected cells is measured. When cells containing the replicon are treated with no dsRNA and no Oligofectamine, or Oligofectamine in the absence of any dsRNA oligonucleotide, a high level of RNA is obtained (0% inhibition). Similar results are obtained when cells are transfected with control dsRNA oligonucleotide LZR-19 in the presence of Oligofectamine. Together, these results demonstrate that neither Oligofectamine nor the control dsRNA oligonucleotide significantly affects the replication of the HCV replicon in the replicon-containing cell line.

In contrast, a significant reduction in RNA level (78-88% inhibition) is observed when replicon-containing cells are transfected with a number of dsRNA oligonucleotides, i.e., LZ NEO-1, homologous to a region of the neomycin resistance gene in the replicon RNA, and LZ-107, LZ-121, LZ-123, LZ-125, LZ-129, LZ-133, and LZ-135, which are homologous to regions of non-structural genes of the HCV genome also present in

replicon RNA, in the presence of Oligofectamine. Individual inhibition values of 78%, 80%, 84%, 85%, and 88% are observed.

Further indication of the potency of the dsRNA oliogonucleotides in inhibiting HCV replicon RNA accumulation is shown by their IC_{50} values in this process as determined by quantitative PCR. The IC_{50} values range from 0.45 to 3.4 nM, with individual values in between of 1.1, 1.2, 1.4, 1.9, and 2.2 nM. These results in the low nanomolar range clearly demonstrate that dsRNA oligonucleotides corresponding to various regions of the HCV genome are highly effective in inhibiting HCV replicon replication, and strongly suggest that dsRNA oligonucleotides have the potential to be effective therapeutics for the prevention or treatment of HCV infections in humans.

Example 2

Effect of Ribonucleotide Substitutions in a dsRNA on Anti-HCV Activity

To determine the effect of single ribonucleotide substitutions at various positions in an anti-HCV replicon dsRNA, single ribonucleotide mismatches are successively introduced at every nucleotide position of dsRNA LZ129 and tested in the assay described in Example 1. For the purpose of the present example, the term "mismatch" refers to substitution of a ribonucleotide in the 5' to 3' strand of a dsRNA (along with a concomitant complementary substitution in the 3' to 5' strand of the same dsRNA) relative to the corresponding ribonucleotide present in the 5' to 3' strand of the HCV replicon target RNA polynucleotide sequence. Modified dsRNAs are obtained from Dharmacon Research, Inc., Boulder, CO.

The ribonucleotide sequence of dsRNA LZ129 and the modifications tested are as follows. Ribonucleotide substitutions in the 5'-3' strand are shown in bold. The nucleotide sequences of the corresponding 3'-5' strands, containing complementary base substitutions, are also shown:

LZ129	5'-	CGCGCACGAUGCAUCUGGC-3'
	3'-	GCGCGUGCUACGUAGACCG-5'
LZ129(C19G)	5'-	CGCGCACGAUGCAUCUGG**G**-3'
	3'-	GCGCGUGCUACGUAGACCC-5'

LZ129(G18C) 5'- CGCGCACGAUGCAUCUGCC-3'
3'- GCGCGUGCUACGUAGACGG-5'

LZ129(G17C) 5'- CGCGCACGAUGCAUCUCGC-3'
3'- GCGCGUGCUACGUAGAGCG-5'

LZ129(U16A) 5'- CGCGCACGAUGCAUCAGGC-3'
3'- GCGCGUGCUACGUAGUCCG-5'

LZ129(C15G) 5'- CGCGCACGAUGCAUGUGGC-3'
3'- GCGCGUGCUACGUACACCG-5'

LZ129(U14A) 5'- CGCGCACGAUGCAACUGGC-3'
3'- GCGCGUGCUACGUUGACCG-5'

LZ129(A13U) 5'- CGCGCACGAUGCUUCUGGC-3'
3'- GCGCGUGCUACGAAGACCG-5'

LZ129(C12G) 5'- CGCGCACGAUGGAUCUGGC-3'
3'- GCGCGUGCUACCUAGACCG-5'

LZ129(G11C) 5'- CGCGCACGAUCCAUCUGGC-3'
3'- GCGCGUGCUAGGUAGACCG-5'

LZ129(U10A) 5'- CGCGCACGAAGCAUCUGGC-3'
3'- GCGCGUGCUUCGUAGACCG-5'

LZ129(A9U) 5'- CGCGCACGUUGCAUCUGGC-3'
3'- GCGCGUGCAACGUAGACCG-5'

LZ129(G8C) 5'- CGCGCACCAUGCAUCUGGC-3'
3'- GCGCGUGGUACGUAGACCG-5'

LZ129(C7G) 5'- CGCGCAGGAUGCAUCUGGC-3'
3'- GCGCGUCCUACGUAGACCG-5'

LZ129(A6U) 5'- CGCGCUCGAUGCAUCUGGC-3'
3'- GCGCGAGCUACGUAGACCG-5'

LZ129(C5G) 5'- CGCGGACGAUGCAUCUGGC-3'
3'- GCGCCUGCUACGUAGACCG-5'

LZ129(G4C) 5'- CGCCCACGAUGCAUCUGGC-3'
3'- GCGGGUGCUACGUAGACCG-5'

LZ129(C3G) 5'- CGGGCACGAUGCAUCUGGC-3'
3'- GCCCGUGCUACGUAGACCG-5'

LZ129(G2C) 5'- CCCGCACGAUGCAUCUGGC-3'
3'- GGGCGUGCUACGUAGACCG-5'

LZ129(C1G) 5'- GGCGCACGAUGCAUCUGGC-3'
3'- CCGCGUGCUACGUAGACCG-5'

In each case, two 2'-deoxythymidine residues are present at the 3' end of each strand of each dsRNA.

The assay is performed as in Example 1.

Results

Single mismatches are introduced at every successive position in the active anti-HCV dsRNA LZ129 to determine their effect on the level of replicon RNA replication in Huh7 cells compared to that of LZ129. Ribonucleotide positions are designated starting from the 5' end of the 5' to 3' sense strand of the dsRNAs. To maintain the percent G + C content of LZ129 in the mismatched dsRNAs, the corresponding ribonucleotide substitution is made in the complementary strand of each dsRNA, i.e., G and C, and A and U, are substituted for one another.

As shown in Figure 1, single mismatches at the 5' end of dsRNA LZ129 are well tolerated at positions 1-6. Mismatches in positions 7-12 decrease, but do not abrogate, inhibition of HCV replicon replication. Like changes at positions 1-6, single mismatches

at positions 13-19 appear to have little effect on the inhibition of replicon replication compared to that caused by LZ129.

These results demonstrate that single ribonucleotide mismatches are tolerated in an anti-HCV dsRNA, and that diminution in anti-HCV replicon activity is more pronounced when such mismatches occur internally rather than toward either end of the dsRNA molecule. Such mismatches can be present in any one of the six terminal ribonucleotides at the 5' or 3' end of a 19 nucleotide duplex. It is fully expected that the same holds true for dsRNA duplexes 20 nucleotides and longer in length as well.

The invention being thus described, it is obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

What Is Claimed Is:

1. An isolated double stranded RNA oligonucleotide about 19 to about 25 ribonucleotides in length,

wherein one strand of said isolated double stranded RNA oligonucleotide comprises the same nucleotide sequence as about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of a region of a hepatitis C virus target RNA polynucleotide sequence required for hepatitis C virus infection, replication, or pathogenesis *in vitro* or *in vivo* in a host cell,

wherein said isolated double stranded RNA oligonucleotide causes inhibition of infection, replication, or pathogenesis of said hepatitis C virus *in vitro* or *in vivo* when introduced into a host cell containing said hepatitis C virus, and

wherein said isolated double stranded RNA oligonucleotide exhibits an IC_{50} in the range of from about 0.0001 nM to about 1 μM in an *in vitro* assay for at least one step in infection, replication, or pathogenesis of said hepatitis C virus, or

a functionally equivalent variant of said isolated double stranded RNA oligonucleotide.

2. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of claim 1, wherein said hepatitis C virus target RNA polynucleotide sequence comprises a region of the RNA genome of hepatitis C virus.

3. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of claim 1 or 2, wherein said hepatitis C virus target RNA polynucleotide sequence codes on expression for a peptide, polypeptide, or protein.

4. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of claim 3, wherein said peptide, polypeptide, or protein is structural or non-structural.

5. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of claim 1 or 2, wherein said hepatitis C virus target RNA polynucleotide sequence encodes an untranslated RNA species.

6. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-5, wherein said isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof modulates transcription, translation, or replication of said hepatitis C virus target RNA polynucleotide sequence.

7. The functionally equivalent variant of any one of claims 1-6, wherein said one strand of said functionally equivalent variant has a sequence identity in the range of from about 80% to about 99% compared to about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of a region of said hepatitis C virus target RNA polynucleotide sequence.

8. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-7, wherein said one strand is the 5' to 3' strand, or the equivalent thereof.

9. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-8, wherein said isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof has a G+C content in the range of from about 30% to about 70%.

10. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-9, which is selected from the group consisting of LZ-107, LZ-121, LZ-123, LZ-125, LZ-129, LZ-133, LZ-135, and mixtures thereof.

11. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-10, wherein said isolated double

stranded RNA oligonucleotide is a duplex about 19 basepairs in length, and comprises a single mismatched ribonucleotide basepair at any one of nucleotide positions 1 to 6 or 13 to 19 compared to any one of corresponding contiguous nucleotide positions 1 to 6 or 13 to 19, respectively, in the 5' to 3' strand of a hepatitis C virus target RNA polynucleotide sequence.

12. The isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-11, wherein said isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof further comprises two overhanging 2'-deoxythymidine residues or two overhanging uridine residues at the 3' terminus of each strand of said isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof.

13. A composition, comprising an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-12, and a buffer, carrier, diluent, or excipient.

14. A pharmaceutical composition, comprising an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-12, and a pharmaceutically acceptable buffer, carrier, diluent, or excipient.

15. Use of an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof according to any one of claims 1-12 to prepare a medicament for the prevention or treatment of hepatitis C virus infection.

16. A method of inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence in a host cell, comprising:

introducing into said host cell an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-12 in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence in said host cell.

17. The method of claim 16, wherein inhibition of the function of said hepatitis C virus target RNA polynucleotide sequence results in inhibition of infection, replication, or pathogenesis of said hepatitis C virus in said host cell.

18. A method of inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence.

19. The method of claim 18, wherein inhibition of the function of said hepatitis C virus target RNA polynucleotide sequence results in inhibition of infection, replication, or pathogenesis of said hepatitis C virus in said host cell.

20. A method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell two or more isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said two or more isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

21. The method of claim 20, wherein inhibition of the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences results in inhibition of hepatitis C virus infection, replication, or pathogenesis in said host cell.

22. A method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in a different region in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

23. The method of claim 22, wherein inhibition of the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences results in inhibition of hepatitis C virus infection, replication, or pathogenesis in said host cell.

24. A method of inhibiting the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences in a host cell, comprising:

introducing into said host cell multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of said two or more hepatitis C virus target RNA polynucleotide sequences,

wherein at least one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of at least one of said two or more hepatitis C virus target RNA polynucleotide sequences, and

at least two of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprise nucleotide sequences homologous to the nucleotide sequences of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of at least one other of said two or more hepatitis C virus target RNA polynucleotide sequences.

25. The method of claim 24, wherein inhibition of the function of said hepatitis C virus target RNA polynucleotide sequences results in inhibition of hepatitis C virus infection, replication, or pathogenesis in said host cell.

26. The method of any one of claims16-25, wherein said host cell is a human liver cell.

27. The method of claim 26, wherein said liver cell is a hepatocyte.

28. The method of any one of claims 16-27, wherein each one of said isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof is introduced into said host cell in an amount effective to completely inhibit the function of the hepatitis C virus target RNA polynucleotide sequence to which it corresponds, or to completely inhibit infection, replication, or pathogenesis of said hepatitis C virus in said host cell.

29. The method of any one of claims 16-28, wherein said host cell is present in a cell culture, a tissue, a tissue explant, an organ, or a human patient.

30. The method of claim 29, wherein said host cell is present within a human patient, and said double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) introduced into a body cavity or interstitial space of said patient.

31. The method of claim 29, wherein said host cell is present within a human patient, and said double stranded RNA oligonucleotide(s) or functionally equivalent

variant(s) thereof is(are) delivered to said host cell via a retroviral or nonviral transfer method.

32. The method of claim 29, wherein said host cell is present within a human patient, and said double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) delivered to said host cell via oral, topical, parenteral, vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration.

33. The method of claim 29, wherein said host cell is present in a cell culture or a tissue explant, and said double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) introduced into said host cell by incubating said cell culture or tissue explant in a solution comprising said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof.

34. A method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient an isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof of any one of claims 1-12 in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

35. A method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of said hepatitis C virus target RNA polynucleotide sequence,

wherein each one of said isolated multiple double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding

contiguous ribonucleotides in different regions in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

36. A method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient two or more isolated double stranded RNA oligonucleotides, two or more functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said two or more isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively,

thereby inhibiting infection, replication, or pathogenesis of said hepatitis C virus in said patient.

37. A method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, multiple functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein each one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of each one of said two or more hepatitis C virus target RNA polynucleotide sequences, respectively.

38. A method of preventing or treating a hepatitis C virus infection in a human patient in need thereof, comprising:

administering to said human patient multiple isolated double stranded RNA oligonucleotides, multiple functionally equivalent variants thereof, or mixtures thereof, of any one of claims 1-12 in an amount effective to inhibit the function of each one of two or more hepatitis C virus target RNA polynucleotide sequences,

wherein at least one of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of at least one of said two or more hepatitis C virus target RNA polynucleotide sequences, and

at least two of said multiple isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof comprise nucleotide sequences homologous to the nucleotide sequences of about 19 to about 25 corresponding contiguous ribonucleotides in different regions in the 5' to 3' strand of at least one other of said two or more hepatitis C virus target RNA polynucleotide sequences.

39. The method of any one of claims 34-38, wherein each one of said isolated double stranded RNA oligonucleotides or functionally equivalent variants thereof is administered to said human patient in an amount sufficient to completely inhibit the function of said hepatitis C virus target RNA polynucleotide sequence(s), or to completely inhibit infection, replication, or pathogenesis of said hepatitis C virus.

40. The method of any one of claims 34-39, wherein said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) introduced into a body cavity or interstitial space of said human patient.

41. The method of any one of claims 34-39, wherein said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) administered via a retroviral or nonviral transfer method.

42. The method of any one of claims 34-39, wherein said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof is(are) administered via oral, topical, parenteral, vaginal, rectal, intranasal, ophthalmic, or intraperitoneal route.

43. The method of any one of claims 34-42, wherein when more than one isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof is employed, said isolated double stranded RNA oligonucleotides or functionally equivalent variants are administered concurrently.

44. The method of any one of claims 34-42, wherein when more than one isolated double stranded RNA oligonucleotide or functionally equivalent variant thereof is employed, said isolated double stranded RNA oligonucleotides or functionally equivalent variants are administered sequentially.

45. The method of any one of claims 34-44, wherein said isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof comprise(s) two overhanging 2'-deoxythymidine residues or two overhanging uridine residues at the 3' terminus of each strand.

46. A cell containing one or more isolated double stranded RNA oligonucleotide(s) or functionally equivalent variant(s) thereof of any one of claims 1-12.

47. The cell of claim 46, wherein said cell is a primate cell.

48. The cell of claim 47, wherein said primate cell is a human cell.

49. The cell of claim 48, wherein said human cell is a liver cell.

50. The cell of claim 49, wherein said liver cell is a hepatocyte.

51. The cell of claim 49 or 50, wherein said cell is present in a cell culture, tissue culture, tissue explant, or human subject.

52. A kit or pharmaceutical pack comprising reagents for inhibiting the function of a hepatitis C virus target RNA polynucleotide sequence required for infection, replication, or pathogenesis of said virus in a host cell, comprising:

a DNA template nucleotide sequence of about 19 to about 25 nucleotides in length also comprising two different promoters selected from the group consisting of a T7 promoter, a T3 promoter, and an SP6 promoter, wherein each promoter is operably linked to said DNA template nucleotide sequence such that two complementary single stranded RNAs are transcribed from said DNA template nucleotide sequence, and wherein one complementary single stranded RNA of said two complementary single stranded RNA molecules comprises a nucleotide sequence homologous to the nucleotide sequence of about 19 to about 25 corresponding contiguous ribonucleotides in the 5' to 3' strand of said hepatitis C virus target RNA polynucleotide sequence;

a plurality of primers for amplification of said DNA template nucleotide sequence;

nucleotide triphosphates for forming RNA;

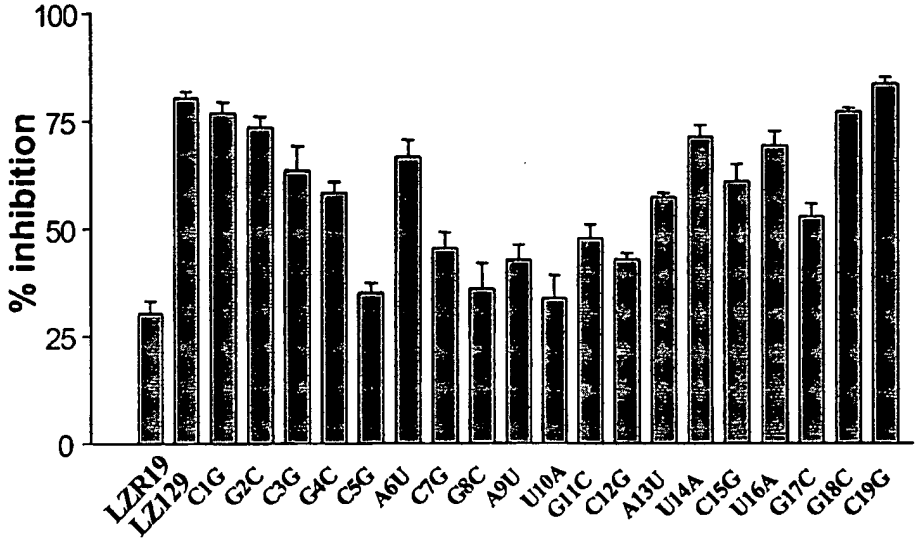
at least two RNA polymerases, each capable of binding to a promoter on said DNA template nucleotide sequence and causing transcription of said nucleotide sequence to which the promoter is operably linked;

a purification column for purifying single stranded RNA;

buffer for annealing single stranded RNAs to yield double stranded RNA; and

RNAse A or RNAse T for purifying double stranded RNA,

wherein said DNA template nucleotide sequence encodes a double stranded RNA oligonucleotide or functionally equivalent variant according to any one of claims 1-12.



LZ129: 5'-CGCGCACGAUGCAUCUGGC-3'

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/21843

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; A01N 43/04; C07H 21/04; A61K 31/07
US CL : 435/6; 514/44; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KATO, J. et al. Interferons Specifically Suppress the Translation from the Internal Ribosome Entry Site of Hepatitis C Virus through a Double-Stranded RNA-Activated Protein Kinase-Independent Pathway. Journal of Infectious Diseases. 2002, Vol. 186, pages 155-163.	1-5
A	MCHUTCHISON, J.G. et al. Hepatic HCV RNA Before and After Treatment with Interferon Alone or Combined with Ribavirin. Hepatology. 2002, Vol. 35, No. 3, pages 688-693.	1-5
X	SAKAMOTO, N. et al. Intracellular Cleavage of Hepatitis C Virus RNA and Inhibition of Viral Protein Translation by Hammerhead Ribozymes. Journal of Clinical Investigation. 1996, Vol. 98, No. 12, pages 2720-2728, see entire document.	1-5
X	WAKITA, T. et al. Antiviral Effects of Antisense RNA on Hepatitis C Virus RNA Translation and Expression. Journal of Medical Virology. 1999, Vol. 57, pages 217-222, see entire document.	1-5

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 2002 (16.09.2002)	26 DEC 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Terra C. Gibbs Telephone No. (703) 308-0196

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PCT/US02/21843

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIMOIKE, T. et al. Interaction of Hepatitis C Virus Core Protein with Viral Sense RNA and Suppression of Its Translation. Journal of Virology. 1999, Vol. 73, No. 12 pages 9718-9725, see entire document.	1-5
Y	LIMA, W.F. et al. Combinatorial Screening and Rational Optimization for Hybridization to Folded Hepatitis C Virus RNA of Oligonucleotides with Biological Antisense Activity. Journal of Biological Chemistry. 1997, Vol 272, No. 1, pages 626-638, see entire document.	1-5
Y	VIDALIN, O. et al. In Vitro Inhibition of Hepatitis C Virus Gene Expression by Chemically Modified Antisense Oligodeoxynucleotides. Antimicrobial Agents and Chemotherapy. 1996, Vol. 40,No. 10, pages 2337-2344, see entire document.	1-5
Y	ALT, M. et al. Core Specific Antisense Phosphorothioate Oligodeoxymucleotides as Potent and Specific Inhibitors of Hepatitis C Viral Translation. Archives of Virology. 1997, Vol. 142, pages 589-599, see entire document.	1-5
Y	MIZUTANI, T. et al. Inhibition of Hepatitis C Virus Replication by Antisense Oligonucleotide in Culture Cells. Biochemical and Biophysical Research Communications. 1995, Vol. 212, No. 3, pages 906-911, see entire document.	1-5
Y	ZHANG, H., et al. Antisense Oligonucleotide Inhibition of Hepatitis C Virus (HCV) Gene Expression in Livers of Mice Infec ted with an HCV-Vaccinia Virus Recombinant. Antimicrobial Agents and Chemotherapy. February 1999, Vol 43, No. 2, pages 347-353, see entire document	1-5
Y	HANECAK, R. et al Antisense Oligonucleotide Inhibition of Hepatitis C Virus Gene Expression in Transformed Hepatocytes. Journal of Virology. August 1996, Vol. 70, No. 8, pages 5203-5212, see entire document.	1-5
A	SMITH, R.M. et al. Secondary Structure and Hybridization Accessibility of Hepatitis C Virus 3'-Terminal Sequences. Journal of Virology. 2002,Vol. 76, No. 19, pages 9563-9574.	1-5
X,P	US 6,423,489 B1 (ANDERSON et al.) 23 June 2002 (23.06.2002), see entire article.	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/21843

Box I Observations where certain claims were f und unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 10
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 10 encompasses sequences that are not in compliance with the sequence rules.

3. ☒ Claim Nos.: 6-52
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.